

In the event of an escape, BCM must deploy maximal effort to recapture any escaped animal. In the highly unlikely event of an escape, animals are expected to stay around the site and easily be recaptured.

If this does not happen then BCM will employ 2 trappers and 1 vehicle full time until issue is resolved and we will cover the reasonable expenses of a Natural Resources ranger who will oversee this operation.

Only if we fail to carry out the above can the government step in and do this and deduct costs on a progressive basis from the *Animal Import Bond*.

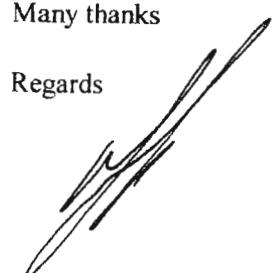
Other permit issues.

There are other important points to resolve in the permit but these are more of an administrative nature and should be easily done so at the same time. These I can send separately if required now. We also need to finalise the EQB permit issues!

Bioculture would appreciate your thoughts on how we may proceed on this matter.

Many thanks

Regards



Owen Griffiths

27.5.2010

Javier Vazquez Morales, Esq.
Executive Director
Puerto Rico Industrial Development Company

Dear Javier,

Trust this finds you well. I am writing to you concerning our Animal Import Permit. While it is certainly a step forward in the sense that a permit has been issued, the conditions applied are clearly un-workable for us as is. I know that our legal team has forwarded to you our comments and suggestion on modifications that will make this workable. It should not be too difficult to amend most of the conditions that need to be amended. Briefly these are summarized here:

Condition 1.5 - Bioculture is authorized to import and export for scientific purposes, up to 4500 Macaca fascicularis during the term of the permit. As we are importing 4,500 animals for breeding in Puerto Rico for sale in the USA and elsewhere, the numbers should refer to imports with no limit on exports.

Condition 1.7.1 - It is illegal to sell, assign or transfer the animals to another person or entity, within the territory or Puerto Rico except to academic institutions (universities) dedicated to the scientific investigation in Puerto Rico. As Puerto Rico wants to encourage a Biomedical Research Industry in Puerto Rico, this should be amended to say:

It is illegal to sell, assign or transfer the animals to another person or entity, within the territory or Puerto Rico, except to those entities authorized by law that are dedicated to scientific investigation in Puerto Rico.

The real project stopper is the issue of the bond: that there is one; the quantum and conditions. This issue had already been the subject of discussions between PRIDCO and the DNR. Our understanding was we had an agreement on this issue and that the liability insurance would suffice. Now they have re-visited this issue. We have for your information invested very heavily in state of the art security. It would also seem to us to be a discriminatory requirement.

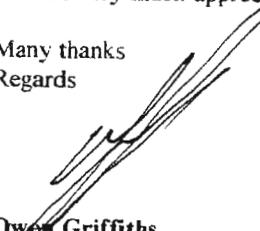
I have a strong feeling that we are almost there! However the bond issue as formulated is just not workable, as I am sure you will agree.

Also we still have the issue of the EQB permit still to resolve.

I would very much appreciate your thoughts on how we can resolve this issue once and for all.

Many thanks
Regards

Owen Griffiths





Bioculture (Puerto Rico) Inc.
P.O. Box 10000, San Juan, PR 00901
Tel: (787) 764-1000

Document BCM.PR.02.07

Animal Trapping Team

Bioculture has over 25 years experience in the trapping of wild (feral) monkeys on the island of Mauritius.

Trapping of monkeys requires a great knowledge of animal biology and behaviour.

The best trapping team should be comprised of an animal care taker who is very familiar with animal behaviour and an assistant to help place and maintain traps.

Equipment needed:

- Binoculars
- 4 individual traps that are baited with food and are triggered by the animal itself. These are placed near animal with its favorite food inside.
- Nets for animal trapping.
- Tranquilizing darts which can be fired or blown at the animal.
- Radio communication
- 4x4 vehicles.

A daily operation cost for a professional trapping team is about 200\$

A monthly operation cost of a professional trapping team is about 5000\$

A trapping team will cover a very large area per day searching for animals.

It should however be remembered that:

1. Animals as a general rule will not venture far from the farm site in the event of an escape, and are thus easily recaptured.
2. Bioculture has invested very significantly in cage design and infrastructure and site fencing to prevent escapes.

Owen Griffiths
Managing Director.





Trappers, Net, dart gun and transport cage



PPI offers two lines of handling gloves to provide your personnel with the best protection against bites and scratches.

Traditional Glove: The cotton-lined glove is made from 2 1/2 - 3 oz. natural cowhide. The safety of the glove is augmented by an 11" above the elbow gauntlet. Made from 2 - 2 1/2 oz. stiff cowhide, the gauntlet is glued and double stitched to the glove.

Traditional Overmit: A 3-finger or 5-finger overmit, made from 2 - 2 1/2 oz. natural cowhide, offers the handler additional protection. Sold separately.

Kevlar Gloves: The hand and finger section is made from 100% cowhide and lined with Kevlar for superior protection and improved dexterity. The full-length cuff is made from split grain cowhide for added flexibility.

Leather Doubler: For the highest level of protection, a 100% cowhide doubler is available with heavy-duty snaps for attachment over the Kevlar Gloves. (Used only with Kevlar Gloves).

ITEM NO.	GLGIVE DESCRIPTION
1HGL00	Traditional Handling Glove (One Size Fits All)
1HGL03	3-Finger Overmit
1HGL05	5-Finger Overmit
1HGL10	Kevlar Handling Glove (Medium)
1HGL20	Kevlar Handling Glove (XL)
1HGL15	Leather Doubler (Medium)
1HGL20	Leather Doubler (XL)

Prima-Carrier

The Prima-Carrier provides a safe and quiet environment for transferring your animals between cages or animal holding rooms. Each box has observation/ventilation holes on the sides. The Prima-Carrier is made from rotational molded polyethylene and can be easily sanitized in cage washers. It is available with two guillotine doors, two side-sliding doors, or combination doors (one guillotine and one side-sliding door). Clear doors sold separately. Weight 10 lbs.

1HTR05	Prima-Carrier (Guillotine Doors)	20 1/4" x 13 1/2" x 16", add 3 1/2" for handle
1HTR10	Prima-Carrier (Side Sliding Doors)	20 1/4" x 13 1/2" x 16", add 3 1/2" for handle
1HTR15	Prima-Carrier (Combination Doors)	20 1/4" x 13 1/2" x 16", add 3 1/2" for handle
1HTR20	S.S. Transfer Box (Combination Doors)	20" x 13" x 16"
1HTRD6	Clear Guillotine Doors	12" x 17"
1HTRD8	Clear-Side Sliding Doors	13" x 15"

Transfer Lift

The Transfer Lift is designed to provide assistance to technicians while moving animals between transfer boxes and the cages. The lift uses weights (5 - 50 lbs) to counter balance the combined weight of the animal and the transfer box, creating a "zero gravity" effect. The lift mechanism locks in position at 1 1/2" increments for added safety and has a lifting range of 12" - 72" above the floor. An optional Lift Adapter (sold separately) is available to configure the Transfer Lift to accept the Prima-Carrier. The lift weighs 210 lbs.

1HTL10	Transfer Lift
1HTL20	Transfer Lift Adapter



PPI is proud to offer the FLEXI-NET line of capture nets. The FLEXI-NET's resilient polycarbonate hoop (or suspension) minimizes harm to animals by absorbing energy and bending when capturing animals against walls, ceilings, and floors. FLEXI-NET's plastic suspension, elastic cord-mounted net, and tempered aluminum handle continues to absorb energy when the netted animal struggles.

The FLEXI-NET is comprised of three components: the handle, the net suspension (the part that holds the mesh bag), and the mesh bag. The modular concept allows you to customize your FLEXI-NET to best meet your needs. Furthermore, each component can be easily removed for cleaning, repair, or replacement when required.

Net Handles

You should choose a handle with the shortest possible length consistent with required reach and the animal's weight.

PART NO.	HANDLE DESCRIPTION	PRIMATE WEIGHT
1H1H30	30" Handle: Light Duty, Double Grip	0 - 3 kg
1H1H36	36" Handle: Medium Duty, Double Grip	3 - 10 kg
1H1H40	40" Handle: Medium Duty, Double Grip	3 - 10 kg
1H1H48	48" Handle: Medium Duty, Double Grip	3 - 10 kg
1H1H53	53" Handle: Heavy Duty, Double Grip, CG (Cushion Grip)	10 - 25 kg
1H1XP1	Extension Handle: 4' - 12', Medium Duty, CG	3 - 10 kg
1H1XP2	Extension Handle: 4' - 8', Heavy Duty, CG	10 - 25 kg

Net Suspension

Net suspension shape and rigidity are the most important choice when customizing your FLEXI-NET. The strength of your net suspension will depend on the primate's weight.

Net suspensions are also offered in three styles: hoop net suspension, penta net suspension, and ogive net suspension (pictured above).

The suspension shape is a matter of personal preference. The hoop suspension is recommended for open areas like field corals. While the penta and ogive suspensions work well in tight spaces.

PART NO.	SUSPENSION DESCRIPTION	CR. & ROD DIA.	PRIMATE WEIGHT
1H2H36	36" Hoop Suspension - Light Duty	36" x 3/8"	0 - 3 kg
1H2H45	45" Hoop Suspension - Medium Duty	45" x 1/2"	3 - 10 kg
1H2P45	45" Penta Suspension - Medium Duty	45" x 1/2"	3 - 10 kg
1H2H54	54" Hoop Suspension - Medium Duty	54" x 1/2"	3 - 10 kg
1H2P54	54" Penta Suspension - Medium Duty	54" x 1/2"	3 - 10 kg
1H2O56	56" Ogive Suspension - Medium Duty	56" x 1/2"	3 - 10 kg
1H2H72	72" Hoop Suspension - Heavy Duty	72" x 5/8"	10 - 25 kg
1H2P72	72" Penta Suspension - Heavy Duty	72" x 5/8"	10 - 25 kg
1H2O84	84" Ogive Suspension - Heavy Duty	84" x 5/8"	10 - 25 kg



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Description

PNEU-DART Model 326B Break Loading Pistol

Model 326B Specifications



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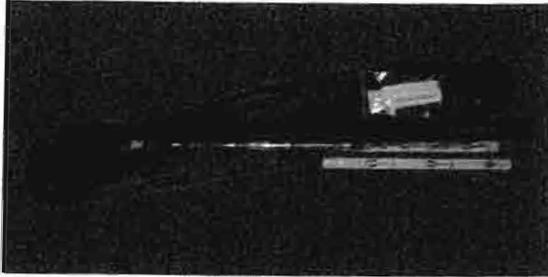
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Blo-Jector Kit



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Link is made for detailed view



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BLO-JECTOR KIT CONTENTS

- 1 x Blo-Jector Kit



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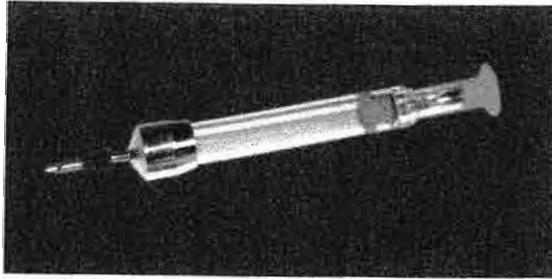
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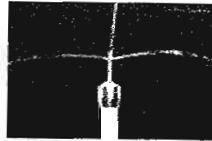
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1.0 CC Type 'P' Dart (5 Pack)



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1.0 CC Type 'P' Dart (5 Pack)

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0.50*

PLEASE NOTE RESTRICTIONS

No Flight Stabilizers

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Description

All darts currently priced here will require a 19 gauge hypodermic fill needle. With each order include **FREE** complimentary 19 gauge fill needle will be located in your shipment.

Poly Dart

1.0 CC Type 'P' Dart (5 Pack)

How They Work



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Pneu-Dart Inc. - The best remote injection equipment and tranquilizer guns for your remote drug delivery needs.

<http://shop.pneudart.com/>

Animal Control 389 Long Range Package



Description

THIS
 ANIMAL CONTROL 389
 LONG RANGE PACKAGE
 INCLUDES THE FOLLOWING:
 1. PNEU-DART 389
 2. PNEU-DART 389
 3. PNEU-DART 389
 4. PNEU-DART 389

Federal Excise Tax.
 This item carries FET. The following
 Federal tax will be applied to your order
 when final processing occurs : \$50.04

Disclaimer

** Sale of the cartridge fired rifles is regulated by Federal Law. Shipment is restricted to military institutions, Sheriff's and Police departments or holders of a Federal Firearm License. If you do not have a license, arrange for a local gun dealer to accept the rifle for you. We must have a signed certified copy of his license on file permitting us to ship the projector directly to the assigned license holder.

Item URL: http://shop.pneudart.com/products/Animal_Control_389_Long_Range_Package-103-30.html



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Animal Control 389 Long Range Package



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Description

Federal Excise Tax:

This item carries FET. The following Federal tax will be applied to your order when final processing occurs - \$50.04

Disclaimer

**Sale of the cartridge fired rifles is regulated by Federal Law. Shipment is restricted to military institutions, Sheriff's and Police departments or holders of a Federal Firearm License. If you do not have a license, arrange for a local gun dealer to accept the rifle for you. We must have a signed certified copy of his license on file permitting us to ship the projector directly to the assigned license holder.



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Animal Control 178B Medium Range Package



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Description

Animal Control 178B Medium Range Package

The Animal Control 178B Medium Range Package is a complete system for controlling animal populations. It includes a projector, darts, and accessories. The projector is designed for medium range use and is easy to operate. The darts are made of a special material that is safe for animals and humans. The accessories include a carrying case and a manual.



Bioculture (Puerto Rico) Inc.
 Pueblita Carmen, Box HCO2 - 4250
 Guayama
 Puerto Rico 00784

Document BCM.PR.01.07

Euthanasia of animals

- ⇒ Animals are euthanized by an injection of concentrated anesthetic.
- ⇒ The Material is given based on the body weight and the quantity for adult animal is 4-5 kg of body weight (like a cat).
- ⇒ The price of a dose to anesthetic to euthanize one animal is between \$1 and \$2.
- ⇒ Procedure to be carried out by veterinarian with animal technician.
- ⇒ A veterinarian can euthanize a very large number of animals per day if needed.
- ⇒ In animal shelters veterinarians can euthanize hundreds of animals per day.
- ⇒ We estimate the cost for one animal with its disposal to be not more then \$50 and the price will drop sharply if the quantity is much bigger.

Cost table for an euthanasia procedure.

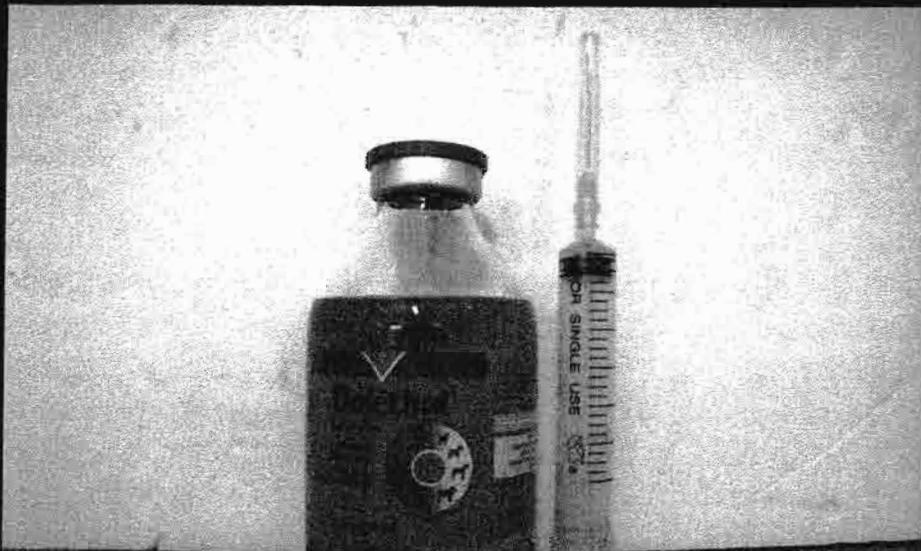
Time/manpower for procedure: 1 animal technician and 1 veterinarian for 2 mins (time taken for procedure)

PROCEDURE: EUTHANASIA

	AMOUNT	COST (\$)	
TIME	2 mins		
MANPOWER			
Handler/Technician	1		
Veterinarian	1		
ITEMS			
Syringe+ Needle	2	0.50	
Latex Gloves	8	0.30	
Ketamine	0.4 ml	0.30	
Dolethal	4 ml	1.00	
Disposal Bag	1	0.50	
TOTAL		\$2.60	

All calculations based on a 4 kg animal
 Disposable items plus medicine costs based on \$US costs in Mauritius.





Doléthai

COMPOSITION

POSOLOGIE

USAGE VÉTÉRINAIRE
A NE DELIVRER QUE SUR ORDONNANCE
NE PAS DELIVRER AU PUBLIC
ADMINISTRATION STRICTEMENT RESERVEE
AUX VÉTÉRINAIRES



KETAMIN 10% INJ

Composition:

Contains per ml solution 100 mg Ketamine (as hydrochloride)

Indications:

Ketamine is used (as monotherapy) for induction of short-term anaesthesia in examinations of restless animals, radiographic procedures and changing of wound dressings. In combination with atropine and xylazine it is used in surgical procedures (ovariectomy, castration, caesarian section, surgery on the jaw, tooth extraction, cleaning of teeth, eye-, nose- and ear surgery, opening of abscesses)

Contra-indications:

Decompensatio cordis, intracranial surgery or skull trauma, kidney and liver insufficiency, glaucoma.

Dosage and administration:

For intramuscular administration:

Dogs: 5-15 mg per kg bodyweight alone or 6-10 mg per kg bodyweight combined with 1-2 mg xylazine per kg bodyweight and atropine 0,05-0,1 mg per kg bodyweight.

Cats: 10-20 mg per kg bodyweight or 8-20 mg per kg bodyweight combined with 1-2 mg xylazine per kg bodyweight and atropine 0,05-0,1 mg per kg bodyweight (s.c.)

Cattle: 2-5 mg per kg bodyweight (i.m.).

Goats: 10 mg per kg bodyweight combined with 0,22 mg xylazine per kg bodyweight (i.m.).

Side effects:

Induction of katelepsia, deep analgesia, amnesia, in which the reflexes of the larynx and pharynx are present. Temporary elevated blood pressure and increased heart rate. During the recovery excitation, tremors and muscle spasms can occur. Breathing disorders. The eyes stay open (dehydration cornea), often a light nystagmus. The muscle tone increases, as a result convulsions and muscle spasms can occur. Increased salivation.

Precautions:

Because of sensitisation and contact dermatitis, direct skin contact must be avoided during administration.

Interactions:

Do not administer simultaneously with choline-esterase inhibitors like organophosphoric compounds.

Withdrawal times: None

Storage conditions:

Store at room temperature (between 15 and 25°C).

Pierced bottle: 30 days if stored cool (between 8 and 15°C) and in the dark.

Packing: Vial of 10 ml or 25 ml; 12 vials in a carton.

B-ENG-01-02 / E-1981 Dutch Farm International BV - Holland

BIOCULTURE (Puerto Rico) WORK INSTRUCTION

Section : WI83-04	Issue No. : 8
Title : Euthanasia, Post-Mortem & disposal of animal carcasses	Date of Issue : 3 April 2009
Compiled by : Senior Veterinarian	Approved by : Vet Manager

Sites applicable: All

Responsibilities: Senior Veterinarian
Vet Staff
Animal Welfare Officer
Senior Animal Technicians
Animal Technician

Policies:

1. Euthanasia is only elected for animals having reached a state of total reject or beyond any reasonable humane treatment.
2. Euthanasia is performed by trained Vet Staff.
3. Post Mortem examination is performed on all dead animals except abortion and records kept.
4. All carcasses are stored in the freezer and removed by a biohazard disposal company.
5. The training manuals TMV11 and TMV31 shall be referred to while implementing this instruction.

	Activities	Responsibilities	Notes	Records
1	<p>Euthanasia.</p> <ul style="list-style-type: none"> - Decision by Vet staff and approval of Senior Vet and Animal Welfare Officer. But in case of emergency situation, the Vet Staff will take decision and inform later the Senior Vet and Animal Welfare Officer. - Vet Staff to ensure animal is unconscious. (Dosage as per WI71-05). - Anaesthesia - Euthanasia: Lethal injection - Confirm death. 	<p>Vet Staff</p>	<p>Done in case of:</p> <ul style="list-style-type: none"> - Total reject. - Beyond reasonable humane treatment. - Slow I.V or I.C done in P.M room: - Phenobarbital (Dolethal) - Dosage : 3-10 ml - No cardiac and pulmonary activity. <p>Not done in presence of other animals.</p>	<p>QR83-16</p> <p>WI71-05 TMV31</p> <p>QR74-25</p>
2	<ul style="list-style-type: none"> - Each animal carcass kept in double and sealed black plastic bags. - Black plastic bag fitted with tag indicating date of death, ID, sex and cage number of the animal. - Black plastic bag containing animal kept in dedicated fridge until transferred to PM room in a dedicated transport box. 	<p>Animal Tech/Vet Staff/Senior Vet</p>	<p>--</p>	<p>--</p>
3	<p>Transfer of corpse:</p> <ul style="list-style-type: none"> - Vet Staff to inform Transport Coordinator. - Necropsy Request to be filled. - inform designated Animal Tech upon arrival at PM Room. - Designated Animal Tech to store carcass in dedicated fridge of PM Room and fill in Post Mortem and Log Book. - Black box to be cleaned, disinfected and returned at latest the next day. 	<p>Vet Staff</p> <p>Vet Staff</p> <p>Driver</p> <p>Designated AT Vet Staff</p>	<p>--</p>	<p>QR63-34</p> <p>IAMS</p> <p>QR83-10</p>

	Activities	Responsibilities	Notes	Records
4	Post Mortem: - Done in PM room only. - Animal euthanised - PM done same day.	Senior Vets/ Designated Vet Staff	- Animal dead on week days: PM done within 24 hours. - Animal dead on week ends: PM done on Monday morning.	--
5	- Perform P.M.	Senior Vets/ Designated Vet Staff	- Follow guidelines for necropsy TMV11 - Collect swabs for analysis if necessary. - Collect any suspect tissues and fix on proper medium for further analysis. - Take photos of unusual findings and send to Vet Manager.	TMV11
6	- Record findings in P.M form.	Vet Staff	- In case of death in hospital, Hospital Record Book is updated with cause of death.	QR83-03 QR83-08 QR83-09
7	- Record organs sampled in Histopathology Request Form	Vet Staff	--	QR83-25
8	- Record in Post Mortem Log Book.	Vet Staff	- Ensure recording done on same day. - Ensure that all data (ID, sex & cage number) pertaining to dead animal are properly recorded.	QR83-10
9	- Clean and disinfect P.M room and working surfaces.	Vet Staff/ Animal Tech	- Virkon & Bleach used (follow TAB64-01). - Send uniforms for disinfection. - Shower compulsory before leaving premises.	TAB64-01 QR64-12

	Activities	Responsibilities	Notes	Records
10	Disposal of carcasses. removed by a biohazard disposal company authorised by PR authorities	Vet Staff Designated Animal Technician	Vet Staff to inform designated Animal Technician. Normally done on same day as P.M. If disposal not possible on same day, carcass placed in red plastic bags to be kept in PM room fridge. Post Mortem Log Book to indicate date of disposal and person responsible.	QR83-10
11	Submit PM form together with treatment form if any to Computer Data Operator within 24 hours.	Vet Staff	Animal History File is updated. PM form also reviewed by Senior Vet.	QR83-08 QR83-09

Records and Specification Table

Record ref	Title
QR63-34	Request for Transport
QR64-12	Cleaning, disinfection and pest control record
QR74-25	Monitoring of controlled substances
QR83-03	Hospital Record Book
QR83-08	Post Mortem Form
QR83-09	Special Necropsy Form
QR83-10	Post Mortem and Log Book
QR83-16	Review of Non Conforming Animals
QR83-25	Histopathology Request
TAB64-01	Cleaning and Disinfection Matrix

Animal welfare issues

1. All euthanasia decisions must be justified.
2. It is the responsibility of the Vet Staff to ensure that if an animal is to be put down, this is done with the highest degree of respect and with an emphasis on making the death as painless and distress-free as possible.

EBI COST ANALYSIS MATRIX 2009

This cost analysis matrix uses actual 2007 cost and animal data from a municipal animal control agency in North Carolina.

- *Note: no actual data for fractious / feral or age breakdown – those data are estimates.*
- Number of dogs euthanized: 2430 (1701 over 4 months – 70% and 729 less than 4 months – 30%) (972 fractious – 40%)
- Number of cats euthanized: 2997 (1798 over 4 months – 60% and 1199 less than 4 months – 40%) (1499 feral – 50%)
- Total dogs and cats euthanized: 5427
- Average number of animals euthanized per day: 15 (5427 / 365 days)

Assumptions:

- a. fractious / feral animals (2471 40% dogs and 50% cats) are given pre-euthanasia anesthesia (ketamine/xylazine)
- b. friendly cats (1498) are given IP injection of sodium pentobarbital with no pre-euthanasia anesthesia
- c. friendly dogs (1215) are given IV injection of sodium pentobarbital with no pre-euthanasia anesthesia

EBI EQUIPMENT COST	
1	Equipment cost: \$670
2	Total equipment cost per animal: \$0.123
EBI LABOR COST	
3	A. # of employees required for IV: 2 B. # of employees required for IP: 1 C. # of employees required for IC: 1 NOTE: 1 employee can safely and effectively administer IP on conscious friendly cats and IC on unconscious or anesthetized animals; 2 employees are required for IV
4	A. # of IV injections (dog): 1215 B. # of IP injections (cat + puppy): 1741 C. # of IC injections (dog + cat): 2471 A. 1215 = 50% of 2430 dogs and 0 cats B. 1741 = 243 puppies (10% of dogs) + 1498 cats (50% of cats) C. 2471 = 972 dogs + 1499 cats

5	Average time to euthanize: 5 minutes average	Transport to euthanasia room + preparation (including IM injection of pre-euthanasia anesthesia as needed) + scanning for microchip + injection + verification of death + removal of carcass + record keeping. <i>NOTE: average time for IP (friendly cats, puppies and kittens) is typically less than 5 minutes; average time for IV is sometimes longer than 5 minutes. Considering an average of 15 animals per day, a typical scenario will involve multiple activities happening concurrently such as animals going under pre-euthanasia anesthesia in a quiet area while another animal is being injected.</i>
6	Total time to euthanize: 75 minutes	# of animals per day (15) × average time to euthanize (5 minutes)
7	Hourly labor cost per worker: \$13.57	Hourly wage: \$10.44 + 30% fringe: \$3.13 = \$13.57
8	5-minute labor cost per worker: \$1.13	Hourly labor cost: \$13.57 / 60 minutes = \$0.226 X 5 minutes = \$1.13
9	IV labor cost: \$2.26	5-minute labor cost: \$1.13 X 2 employees = \$2.26
10	IP and IC labor cost: \$1.13	5-minute labor cost: \$1.13 X 1 employee = \$1.13
11	Total annual IV labor cost: \$2746	IV labor cost: \$2.26 X 1215 = \$2746
12	Total annual IP & IC labor cost: \$4759	IP and IC labor cost: \$1.13 X 1741 (IP) + 2471 (IC) = 4212 X \$1.13 = \$4759
13	Total annual labor cost for IV, IC & IP: \$7505	Labor cost IP & IC + IV = \$2746 + \$4759 = \$7505
14	Total labor cost per animal: \$1.38	Total annual labor cost / # of animals euthanized: \$7504 / 5427 animals = \$1.38
EBI SUPPLY COST		
15	Sodium pentobarbital cost per 250 ml bottle: \$46.00	
16	Cost per ml (cc): \$0.184	Cost of bottle (\$46.00) ÷ 250 ml
17	Average IV dose (dog): 5 ml	50-pound dog average
18	Sodium pentobarbital cost per IV dose:	Cost per ml \$0.184 X average dose: 5 ml

	\$0.92		
19	Annual sodium pentobarbital IV cost: \$1,118	Average IV dose (5 ml) cost: \$0.92 X 1215 dogs = \$1,118	
20	Average IP dose per cat + puppy = 2 ml	7 pound cat and puppy average (some cats and puppies will weigh more, kittens and neonates will weigh less)	
21	Sodium pentobarbital cost per IP dose: \$0.368	Cost per ml \$0.184 X average dose: 2 ml	
22	Annual sodium pentobarbital IP cost: \$641	243 puppy IP + 1498 cat IP = 1741 X \$0.368 = \$641	
23	Average IC dose (dog) = 5 ml	50-pound dog average	
24	Sodium pentobarbital cost per IC dose (dog): \$0.92	Cost per ml \$0.184 X average dose: 5 ml = \$0.92	
25	Annual sodium pentobarbital IC (dog) cost: \$894	Average IC dose cost: \$0.92 X 972 dogs = \$894	
26	Average IC dose (cat) = 1 ml	7-pound cat average (some cats will weigh more, some will weigh less)	
27	Sodium pentobarbital cost per IC dose (cat): \$0.184		
28	Annual sodium pentobarbital IC (cat) cost: \$276	1499 (feral) cat estimate X \$0.184 = \$276	
29	Total sodium pentobarbital cost: \$2,929	Annual IV (\$1,118) + IP (\$641) + IC cat (\$276) + IC dog (\$894) = \$2,929	
30	Average sodium pentobarbital cost per animal: \$0.54	Total sodium pentobarbital cost (\$2,929) / # of animals euthanized (5427) = \$0.54	
31	Syringe cost per animal: \$0.019	Syringe (6 ml) cost: \$19 per 100 (\$0.19 each) estimate 100 uses per syringe (reusing syringes is a standard practice in EBI)	
32	Total annual syringes: 79	Total animals: 5427 EBI injections + 2471 (pre-euthanasia IM injections) = 7,898 injections total / 100 = 79 syringes	
33	Annual syringe cost: \$15.01	79 syringes X \$0.19 = \$15.01	
34	Average syringe cost per animal: \$0.003	\$15.01 / 5427 (total animals euthanized) = \$0.003	
35	Needle cost: \$0.01	Needle (22 ga.) cost: \$10.00 per 100 (one use only)	

36	Total annual needles: 7898	1 per euthanasia: 5427 + 1 per pre-euthanasia anesthesia: 2471 = 7898
37	Annual needle cost: \$78.98	7898 X \$0.01 = \$78.98
38	Average needle cost per animal: \$0.014	\$78.98 / 5427 (total animals euthanized) = \$0.014
39	Pre-euthanasia anesthesia cost per dog: \$1.00	5:1 ratio ketamine/xylazine per 50 pound dog = \$0.40 ml X 2.5 ml = \$1.00
40	Annual pre-euthanasia anesthesia cost for dogs (fractious): \$972	972 fractious dogs X \$1.00 per dog (average weight = 50 pounds, 2.5 ml @ \$0.40 per ml)
41	Pre-euthanasia anesthesia cost per cat: \$0.20	5:1 ratio ketamine/xylazine per 10 pound cat = \$0.40 ml X 0.5 ml = \$0.20
42	Annual pre-euthanasia anesthesia cost for cats (feral): \$299	1499 feral cats X \$0.20 per cat (average weight = 10 pounds, 0.5 ml @ \$0.40 per ml)
43	Annual total cost of pre-euthanasia anesthesia: \$1271	Annual cost dogs (\$972) + cats (\$299) = \$1271
44	Average pre-euthanasia cost per animal: \$0.23	\$1271 / 5427 (total animals euthanized) = \$0.23
45	Total supply cost per animal: \$0.787	Sodium pentobarbital per animal: \$0.54 + syringe: \$0.003 + needle: \$0.014 + pre-euthanasia anesthesia: \$0.23 = \$0.787
EBI TOTAL COST		
46	Total EBI cost per animal: \$2.29	Equipment cost per animal: \$0.123 + labor cost per animal: \$1.38 + supply cost per animal: \$0.787 = \$2.29

CARBON MONOXIDE COST ANALYSIS MATRIX 2009

This cost analysis matrix uses actual 2007 cost and animal data from a municipal animal control agency in North Carolina. Although the actual agency reported best practices use of euthanasia by injection (EBI) for animals younger than 4 months of age (25% of total animals euthanized), this cost analysis assumes 100% chamber use in order to more accurately reflect the industry as a whole and to provide a more useful cost comparison to EBI. Although frequent, 100% chamber use is NOT acceptable practice. Industry standards demand the use of EBI for animals less than 4 months of age and for animals suffering from respiratory conditions, generally poor health or severe injury.

Industry standards recommend administering 0.5 mg / pound acepromazine maleate (tranquilizer) to adult dogs 20 minutes prior to placing them in the chamber to reduce vocalization/agitation. The dose is typically 25 mg for an average 50-pound dog.

- Total number of dogs euthanized: 2430
- Total number of cats euthanized: 2997
- Total number of dogs and cats euthanized: 5427
- Average number of dogs and cats euthanized per day: 15 (365 days)
- Number of employees (operators): 2 (alternate costs for 1 operator are included)
- For purposes of this cost analysis matrix, an average dog is 50 pounds.

CARBON MONOXIDE EQUIPMENT COST	
1	CO chamber: \$10,500 Cutting Edge Fabrication, estimated usable life: 10 years
2	CO sensor: \$300 Unknown brand, estimated usable life: 10 years
3	Chamber lifetime routine maintenance: \$5,000 Estimated cost to maintain seals, gaskets and hardware over 10 years = \$500 per year

4	Annual depreciation: \$1,080	Chamber: \$10,500 + sensor: \$300 = \$10,800 / 10 = \$1,080
5	Annual depreciation + maintenance: \$1,580	Equipment depreciation: \$1,080 + maintenance: \$500 = \$1,580
6	CO equipment cost per animal: \$0.29	\$1,580 (annual depreciation/maintenance) / 5427 (total animals euthanized per year) = \$0.29
	TRANQUILIZER COST	
7	Acepromazine tranquilizer per average dog: \$1.00	Average dog: 50 pounds: 2.5 ml at \$0.40 per ml = \$1.00
8	Syringe / needle cost per dog: \$0.013	Syringe (reused) cost: \$0.003 + needle cost: \$0.01 = \$0.013
9	Tranquilizer cost per 50 pound dog: \$1.013	\$1.00 + \$0.013 = \$1.013
10	Number of dogs tranquilized (estimate): 1701	1701: number of estimated adult dogs euthanized by CO chamber (70% of total dogs)
11	Total annual cost of tranquilizer: \$1,723.11	1701 dogs X cost per dog: \$1.013 = \$1,723.11
12	Tranquilizer cost per animal: \$0.32	\$1,723.11 / 5427 (total animals euthanized) = \$0.317
CO LABOR COST		
13	Number of employees to euthanize: 2	Note: actual municipal animal control agency uses 2 operators (employees) to euthanize by carbon monoxide
14	Load time: 10 minutes	Includes transport to chamber
15	Run time: 35 minutes	Employees do paperwork and watch chamber
16	Unload time: 5 minutes	Remove carcasses, clean chamber for next cycle
17	Total cycle time: 50 min	Load: 10 + run: 35 + unload: 5 = 50 minutes
18	Number of dogs or cats per cycle: 6	Dogs and cats are not mixed in a cycle

19	Number of cycles per day: 2.5	Average number of animals euthanized per day: 15 / number of animals per cycle: 6 = 2.5
20	Total time per day: 125 minutes	Load + run + unload (cycle time) = 50 minutes X 2.5 cycles = 125 minutes (2.08 hours)
21	Labor cost per minute per person: \$0.226	Hourly wage: \$10.44 + 30% fringe: \$3.13 = \$13.57 / 60 minutes = \$0.226
22	Total labor cost per minute (2 operators): \$0.452	\$0.226 X 2 operators = \$0.452
23	Total labor cost per cycle: \$22.60	\$0.452 X 50 minutes = \$22.60
24	Labor cost per day: \$56.25	\$22.60 X 2.5 cycles = \$56.50
25	Labor cost per animal: \$3.77	\$56.50 / 15 animals = \$3.766
26	Alternate: labor cost per animal with 1 operator rather than 2: \$1.88	\$0.226 X 50 minutes = \$11.30 X 2.5 cycles = \$28.25 / 15 animals = \$1.88 (note: will likely take longer to load and unload but is not reflected in this matrix)
	CO SUPPLY COST	
27	CO gas cylinder: \$219.00	Includes cylinder rental plus gas
28	Annual number of cylinders: 15	Total number of cylinders used in 2007
29	Total gas cost:	\$219 per cylinder X 15 cylinders = \$3285
30	Gas cost per day:	Annual cost / 365 days = \$9.00
31	Supply cost per animal: \$0.60	\$9.00 / 15 animals = \$0.60
CARBON MONOXIDE TOTAL COST		

32	CO cost per animal: \$4.98	Equipment cost per animal: \$0.29 + tranquilizer cost: \$0.32 + labor cost per animal: \$3.77 + supply cost per animal: \$0.60 = \$4.98
33	Alternate CO cost (1 operator): \$3.09	Equipment cost per animal: \$0.29 + tranquilizer cost: \$0.32 + labor cost per animal: \$1.88 + supply cost per animal: \$0.60 = \$3.09

CARBON MONOXIDE Vs EBI

34	EBI cost per animal:	\$2.29
35	CO cost per animal (2 operators)	\$4.98
36	CO cost per animal (2 operators) <i>without tranquilizer</i>	\$4.66
36	CO cost per animal (1 operator)	\$3.09
37	CO cost per animal (1 operator) <i>without tranquilizer</i>	\$2.77

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MI 2010 - Humane Euthanasia / Anti-Pound Seizure



Help protect Michigan's shelter animals from inhumane euthanasia practices and Class B Dealer pound seizure!

The Michigan House of Representatives is considering two groundbreaking bills to protect animals.

Pound Seizure

House Bill 4663, or Koda's Bill, would eliminate Class B Dealer pound seizure. Pound seizure is the practice of allowing shelter cats and dogs to be used in experimental research.

"Koda's Bill" is named after a shelter dog who, instead of being placed for adoption, was sold to a USDA Class B Dealer (animal broker) and resold to the University of Michigan, where he was used in the university's Advanced Trauma Life Support Class, and then euthanized. Koda's former family believed that taking him to a shelter would allow him another opportunity to find a home and did not know he would be used in a research experiment.

Companion animals depend on humans for their safety and well-being. Tragically, this dependency is betrayed when shelters allow these pets to be taken by Class B Dealers for resale to research facilities. When Class B dealers and research facilities can obtain cats and dogs like Koda from animal shelters, it diminishes the shelters' credibility and purpose, and betrays public trust.

Currently, two shelters in Michigan practice pound seizure. Passing this bill is essential toward ensuring that the law supports the community's overwhelming rejection of this practice. Moreover, Koda's Bill would lessen the burden on the USDA, since the USDA expends significant resources to frequently inspect the activities of Class B dealers due to prior prosecutions regarding pet theft.

Koda's Bill is sponsored by Rep. John Espinoza and co-sponsored by Reps. Terry Brown, Mike Huckleberry and Jeff Mayes. It was drafted by American Humane and the State Bar of Michigan Animal Law Section.

Humane Euthanasia

House Bill 6042/6043 would ensure that when the state's unwanted, sick or unadoptable shelter animals have to be euthanized, the procedure will only be done by injection of sodium pentobarbital. This method is called "euthanasia by injection" or "EBI."

American Humane considers EBI to be the only acceptable and humane means of euthanasia of dogs and cats in animal shelters.

Most shelter workers wish to hold and comfort a frightened animal in its final moments of life. That act may be the only kindness the animal has ever known. In contrast, even with vigilant oversight, euthanizing any animal by means of a carbon monoxide or dioxide gas chamber is severely inhumane to medium and large dogs, and is demoralizing to the shelter workers. Such outdated practices also create public outcry and demean the purpose of an animal shelter.

American Humane recently commissioned a study on the costs of EBI and gas chambers that proves EBI is *less costly* to communities. Using data from an animal sheltering organization,

TAKE ACTION

Ask your representative to
[protect Michigan's shelter
animals](#)

Make a difference today.

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the study shows that the cost to use carbon monoxide poisoning is \$4.98 per animal. The cost to use EBI, however, is only \$2.29 per animal:

The continual public outcry against the inhumane techniques still used in 11 of the state's shelters illustrates a critical need for HB 6042/6043 to pass.

HB 6042/6043 are sponsored by Reps. Rick Jones and Fred Miller and are modeled after legislation drafted by the American Humane Association with the assistance of the State Bar of Michigan Animal Law Section.

In order to prevent opposition, the committee voted to pass the gas chamber bill with two amendments: one that states the bill does not affect farm animals, and another that lists a January 1, 2012 effective date. Also to prevent opposition, the committee voted to amend the pound seizure bill to allow research facilities to obtain stray animals. While these amendments are disappointing, American Humane is pleased that the bills eventually will finally prohibit gas chambers and Class B Dealers, provisions that are huge steps forward toward making Michigan a more humane state for shelter dogs and cats. Both bills passed the House Agriculture Committee on July 1 and are now headed for a House Floor vote. Please [ask your representative to support them](#).

This action alert is for residents of the following states only: Michigan



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Pets - Shelters, Pounds, And Euthanasia

Despite the popularity of pets, millions of them wind up in public and private shelters each year. The vast majority are dogs or cats. They are turned in by owners who no longer want them or are picked up as strays. Some are lost pets that can be reunited with their owners, but many are homeless animals with no place to go. According to information on the Web site of the Humane Society of the United States (HSUS) in 2005, there are 4,000–6,000 shelters operating in the United States that receive six to eight million dogs and cats each year. Approximately half of these animals are euthanized (killed). The remainder are adopted or reclaimed by owners.

History

According to the HSUS, the first public "pounds" were constructed during the 1700s to impound stray livestock. As American society changed from rural to urban, these facilities switched their focus to stray dogs and became dog pounds. Rabies was a serious public health threat well into the twentieth century, with thousands of cases reported each year. The vast majority of cases were contracted by people from domestic dogs, so animal control departments operated dog pounds as part of public health and safety programs. Their mission was to protect people rather than to ensure the welfare of the dogs.

Following World War II (1939–45), rabies vaccinations for dogs became mandatory in the United States. This program, combined with effective stray dog control, dramatically reduced the occurrence of rabies in dogs. The New York State Health Department recorded up to 200 rabid dogs in the state each year between 1925 and 1944, but by 1959 that number had dropped to twenty to fifty each year, and by 1989 dog rabies had been virtually eliminated in the state. Similar results were obtained across the country. Pounds continued to pick up stray dogs (and cats, by this time) but did so more to control aggressive and nuisance animals and "clean the streets" than as rabies protection. Pounds also became centralized facilities for people to get rid of unwanted pets.

Dog pounds came to be called animal shelters. They held stray animals for a few days (if there was room) to see if their owners would reclaim them. If not, unwanted strays and pets were sold to research laboratories or given to anyone who wanted them. Unclaimed and unplaced animals were killed using whatever means were available. Public animal shelters received little funding from local governments, and humane treatment and euthanasia were not a priority in most jurisdictions.

This began to change as the animal welfare movement gained momentum during the 1960s and 1970s. Shelters came under increasing pressure to focus on welfare issues in addition to public health and nuisance concerns. Some municipal governments began contracting their shelter operations to nonprofit animal welfare organizations, like local humane societies and rescue groups. These organizations held fund-raisers and were able to secure private donations to help shelters operate. However, most shelters continued to euthanize large numbers of animals as the homeless animal population surged out of control.

TABLE 9.1
Shelter euthanasia of owned animals, 1973,
1982, 1992, and 2000

SOURCE: Deborah J. Salem, and Andrew N. Rowan, "Table 1. Shelter Euthanasia of Owned Animals," in *The State of the Animals: 2001*, Humane Society of the United States, 2001

Year	Total owned dogs and cats	Euthanized	Approximate % of owned animals euthanized
1973	65 million	13.5 million	21.0
1982	92 million	8–10 million	10.0
1992	110 million	5–6 million	5.5
2000	120 million	4–6 million	4.5

Euthanasia

The word "euthanasia" comes from a Greek term meaning "good death." During the 1800s, it was first used to describe mercy killing conducted with the approval of the law. In the twentieth century euthanasia of shelter animals was conducted on a massive scale.

Shelter euthanasia rates are tracked nationally by the HSUS and a group called Animal People. Animal People was founded in 1992 and publishes information about animal protection groups worldwide. In general, euthanasia rates have been dropping since the late twentieth century. The HSUS reports that the number of euthanized cats and dogs has dropped considerably in the United States, from about 13.5 million deaths per year in 1973 to four to six million deaths in 2000, while over the same period the total number of cats and dogs has nearly doubled. (See Table 9.1.) In early 2005 the HSUS Web site estimated that three to five million dogs and cats were euthanized annually in American shelters.

Statistics from Animal People are provided in Figure 9.2. They estimate that 4.2 million shelter animals were euthanized in 2002, a record low. The group bases its estimates on data collected from individual cities or states around the country. In 2002 data collected represented nearly 40% of the U.S. human population.

According to Animal People there are regional differences in the data. In general, shelters located in the Northeast have the lowest euthanasia rates, while shelters in the Southeast have the highest rates. This is attributed to several factors, including the weather, the availability of low-cost spay-neuter programs, and animal control policies. The cold winters in the Northeast lower the fertility rates of dogs and cats and claim the lives of stray animals so that fewer end up in shelters to begin with. Animal welfare organizations are much more predominant in the Northeast and provide low-cost spay-neuter programs that help keep down populations of unwanted animals. Many Northeastern municipalities charge pet owners licensing fees with higher amounts for unfixed animals. This is far less common in the South.

Euthanasia Methods

Although the public assumes that animals euthanized at shelters are killed by lethal injection, this is not always true. The AVMA maintains a list of approved euthanasia methods for various types of animals. According to the AVMA, "Euthanasia techniques should result in rapid loss of consciousness followed by cardiac or respiratory arrest and the ultimate loss of brain function. In addition, the technique should minimize distress and anxiety experienced by the animal prior to loss of consciousness." However, the AVMA admits that "the absence of pain and distress cannot always be achieved."

Acceptable euthanasia methods for dogs and cats include intravenous injection of barbiturates (such as sodium pentobarbital or secobarbital) or potassium chloride/anesthetic or gassing the animals with inhalant anesthetics (such as ether), carbon dioxide, or carbon monoxide gas. In addition, gassing with nitrogen or argon is considered acceptable with some reservations on dogs and cats, as are the use of electrocution and penetrating captive bolts (bolts shot at point-blank range from a gun into the animal's skull, which if shot at the proper location destroy enough brain tissue to kill the animal instantly) on dogs only. Each of the methods, along with its advantages and disadvantages, is described in the "2000 Report of the AVMA Panel on Euthanasia" published in the *Journal of the American Veterinary Medical Association* on March 1,

Animal Care & Use Program



Animal Use Protocol

(Click above for template of animal use application)

New Protocols

(Deadline: 5 PM on 1st Monday)
August 2, 2010

Amendments

(Deadline: 5 PM on...)
July 22, 2010

General Program Info
Protocol Development
Forms and Reports
Policies
Guidelines & Suggestions
Animal Care SOPs
Animal Biosafety
Training
Compliance
Animal Diseases
Animals on the Web
Program Contacts
References
Reporting Concerns
EMERGENCIES

Guidelines for Euthanasia (BY AGENT)

Duke University adheres to the guidelines of the American Veterinary Medical Association. A copy of this report is available in pdf format by clicking here. You will need Adobe Acrobat Reader to open this file. The Reader program is a FREE program. Click here to download the Adobe Acrobat Reader.

- Inhalant Anesthetics
- Non-Anesthetic Gases
- Pharmacological Agents
- Physical Methods

Inhalant Anesthetics

In the liquid state, most inhalant anesthetics are topical irritants; therefore, animals should be exposed only to the vapors of the anesthetic. When provided via a few drops of an anesthetic agent on a cotton sponge, which **MUST** be kept separate from the animals by a wire screen or other device to prevent animal contact with the anesthetic agent.

Air or oxygen must be provided during the induction period. All agents are given "to effect" until respiratory and cardiac arrest occurs.

Sevoflurane, halothane, and isoflurane all have a rapid action. Sevoflurane is the most expensive, but is very quick. Halothane is less expensive and a good alternative. Although these are very safe agents, care should be taken to minimize personnel exposure to vapors.

Ether is acceptable but not recommended because it poses an explosive hazard and is a respiratory irritant that is considered stressful to animals. Administration should be performed in a fume hood, and signs indicating that

ether is present or in use should be posted. To avoid explosions, the carcasses of ether-killed animals should be stored in explosion-safe refrigerators or freezers, and should not be incinerated until the ether is removed by aeration in a hood.

Non-Anesthetic Gases

Carbon dioxide (CO₂) is a common agent used for euthanizing rodents and other small animals. Use of a sealed chamber filled by a compressed gas cylinder is required. CO₂ generated by other methods, such as from dry ice, is unacceptable because gas flow can't be regulated precisely. Chambers should not be overcrowded. A CO₂ concentration of 70% or more should be utilized for euthanasia. Because CO₂ can act as a reversible anesthetic, it is imperative that the animals be kept in the chamber for several minutes after respiratory arrest. A secondary method of euthanasia is REQUIRED whenever CO₂ is used. Due to physiologic characteristics, neonates require prolonged exposure to the gas (euthanasia of neonates using non-anesthetic gases is not acceptable at Duke).

Pharmacological Agents

Barbiturates: Barbiturates such as pentobarbital are acceptable for mammalian species and birds. These drugs should be administered intravenously (IV) except in rodents where intraperitoneal (IP) administration is an acceptable alternative. Sodium pentobarbital (Nembutal) is the most common barbiturate agent for euthanasia. The dosage is usually at least twice that required for anesthesia, ranging from 85 mg/kg for larger species to 200 mg/kg for some rodents. The Duke standard for euthanasia with barbiturate is 250 mg/kg for all species. Commercial barbiturate euthanasia formulations as are also appropriate, and should be used following label directions (e.g., 1 ml/10 lb BW for Beuthanasia-D*). Sodium pentobarbital is a Class II drug which is regulated by the Drug Enforcement Agency. Personnel using this agent are required to have a federal AND state DEA license, store it in a locked location, and maintain records which include the date and amount of use.

Chloral Hydrate: Chloral hydrate is not recommended, but may be used in ruminants and swine when administered I.V. at 900 mg/kg, but only after sedation with another drug.

Mag Sulfate or Potassium Chloride: Neither magnesium sulfate nor potassium chloride (KCl) can be used as a sole agent of euthanasia. Overdose with KCl is permissible in an anesthetized animal. Concentrated KCl should be given rapidly IV until rising serum potassium levels result in cardiac arrest.

MS 222: Tricaine methane sulfonate (MS222) can be used either as an injectable agent (200-300 mg/kg of a 1% buffered solution) or as an immersion bath (2 mg/ml in H₂O) for amphibians and fish. The immersion time needed to assure death can range from 20 minutes to three hours, so it may be advantageous to use MS222 as an anesthetic followed by a physical method of euthanasia. Benzocaine immersion (100-200 mg/liter H₂O) is

Bioculture PR - Safety and security

Security and safety

Farm will be surrounded with a 7 feet fence /electronic fence with touch sensors and with security forces on site 24 hours 365 days a year.

Our animal security systems and SOP's for the prevention of animal escape, form part of our internationally accredited Quality System and are a fundamental part of our operations.

We have **AAALAC accreditation and ISO certification**

Animal Security systems

Our animal security systems for the prevention of animal escape operate on a number of levels:

- Staff training and inspections
- Cage design and engineering - Hurricane proof cages.
- Provision of appropriate security equipment & systems.

Safety and Security measures

- ★Training of workers on security SOPs.
- ★Daily cage inspection and supervision.
- ★Site with 24 hour security and surveillance.
- ★Handling equipment
- ★Electric fencing around the farm.
- ★Triple door system on all cages.
- ★Monkey proof latches and locks on all doors.
- ★Hurricane Proof Cages
- ★Cages especially designed & engineered to be Hurricane proof.
- ★Double Electric Fence with motion sensors
- ★Motion sensors

Responsibility

The proposed investment by Bioculture in the establishment of a monkey breeding and rearing farm in Puerto Rico is being made because the demand for Mauritian origin SPF monkeys in the USA is high and is expected to grow in the coming decades.

However in order to further demonstrate our faith in this industry and its future in Puerto Rico and to allay some concerns expressed about what would happen if demand for Mauritian monkeys was to disappear, Bioculture (PR) Inc. is prepared to make a formal undertaking to guarantee to the Gov. of Puerto Rico that should Bioculture wind up its operations in Puerto Rico, it will remove all its animals from the facility at that time.

Bioculture Puerto Rico Inc.

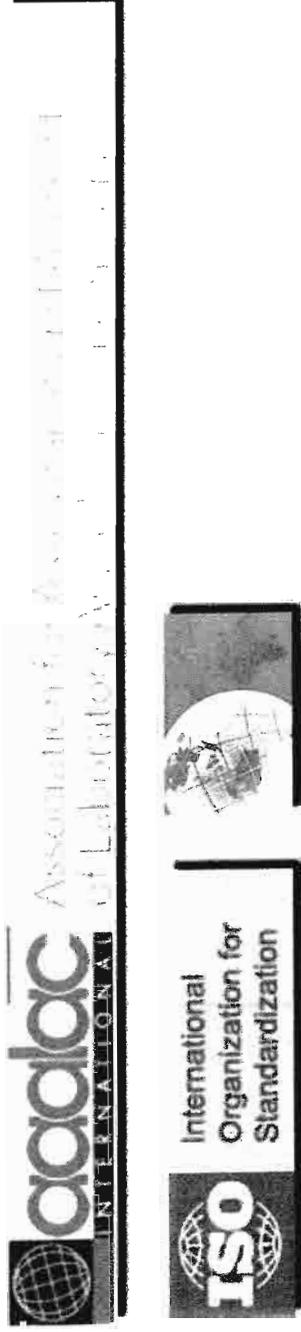


Safety and security

Leading in Safety & Security

Our animal security systems and SOP's for the prevention of animal escape, form part of our internationally accredited Quality System and are a fundamental part of our operations.

We have AAALAC accreditation and ISO certification



Animal Security systems

Our animal security systems for the prevention of animal escape operate on a number of levels:

- Staff training and inspections
- Cage design and engineering - Hurricane proof cages.
- Provision of appropriate security equipment & systems.

Safety and Security measures

Training of workers on security SOPs.

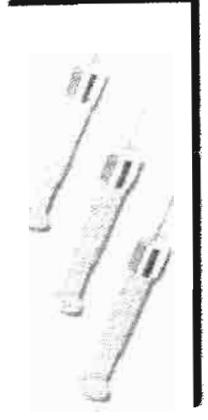
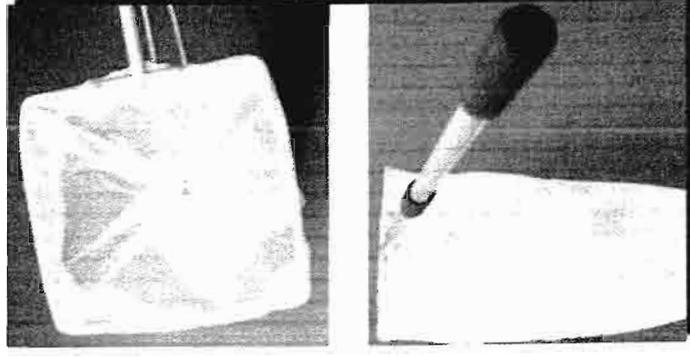
Daily cage inspection and supervision.

Site with 24 hour security and surveillance.



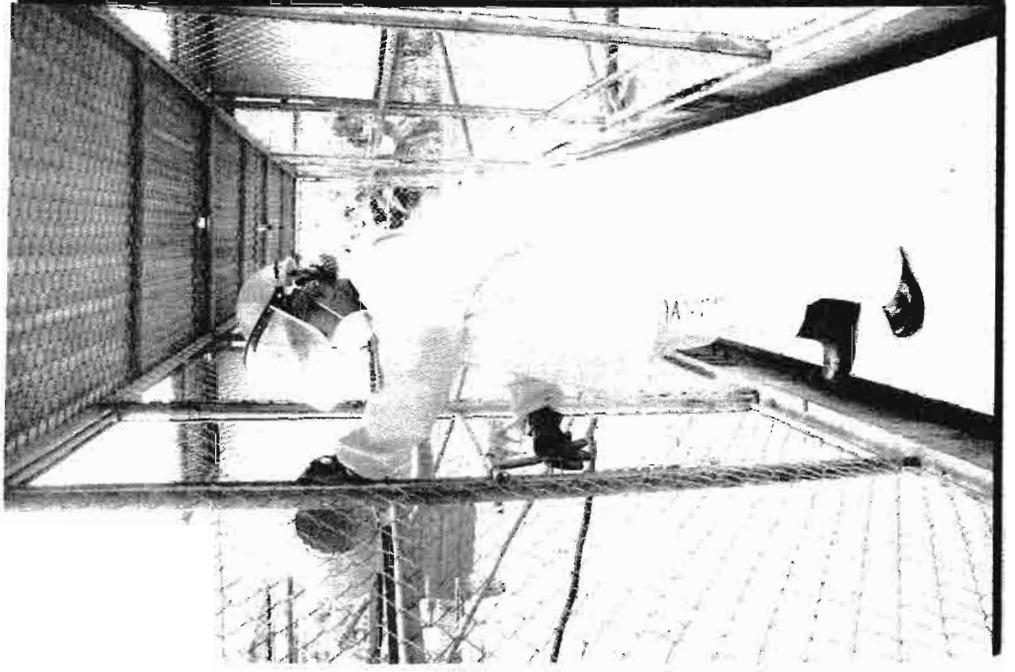
Handling equipment

Electric fencing around the farm.



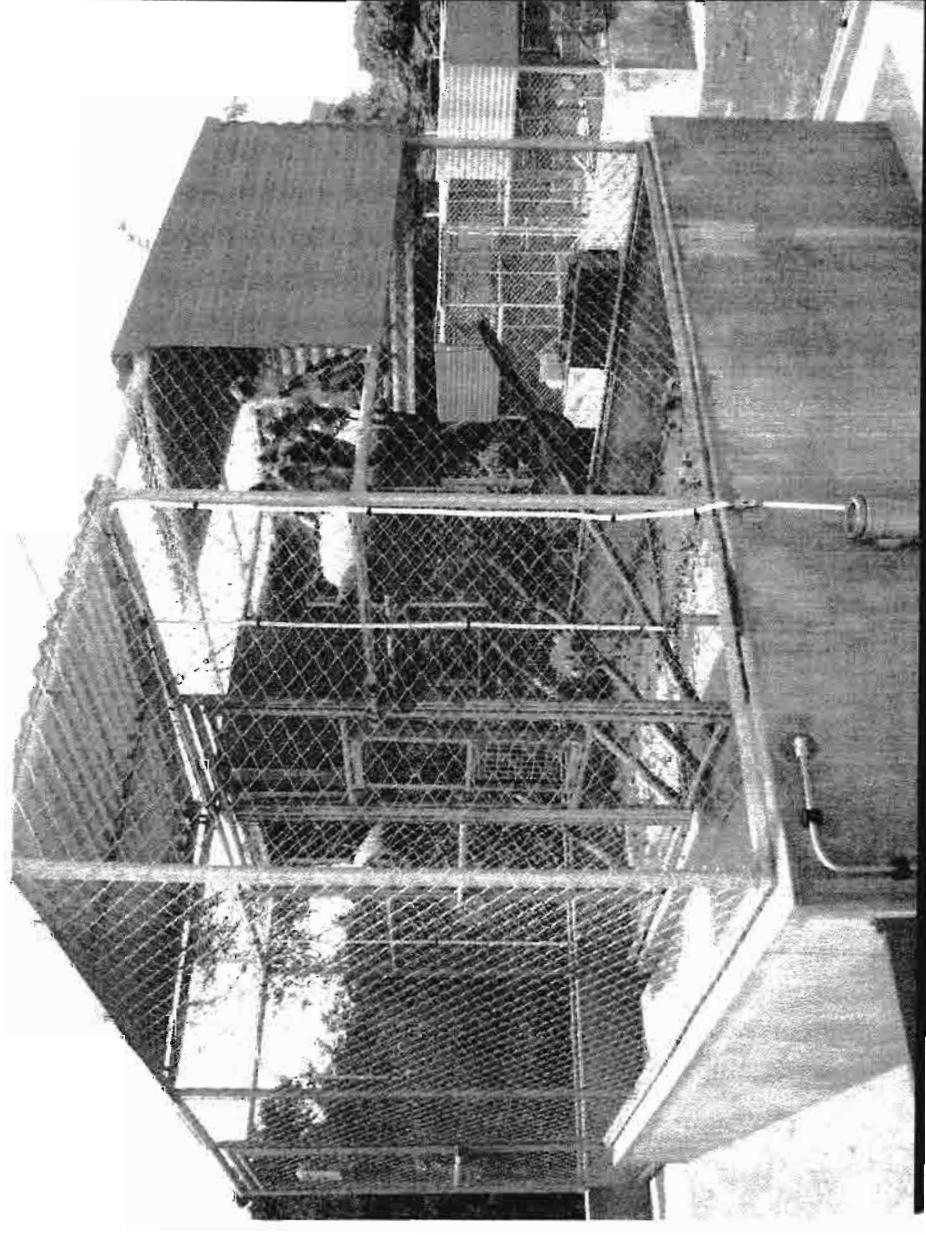
Triple door system on all cages.

Monkey proof latches and locks on all doors.



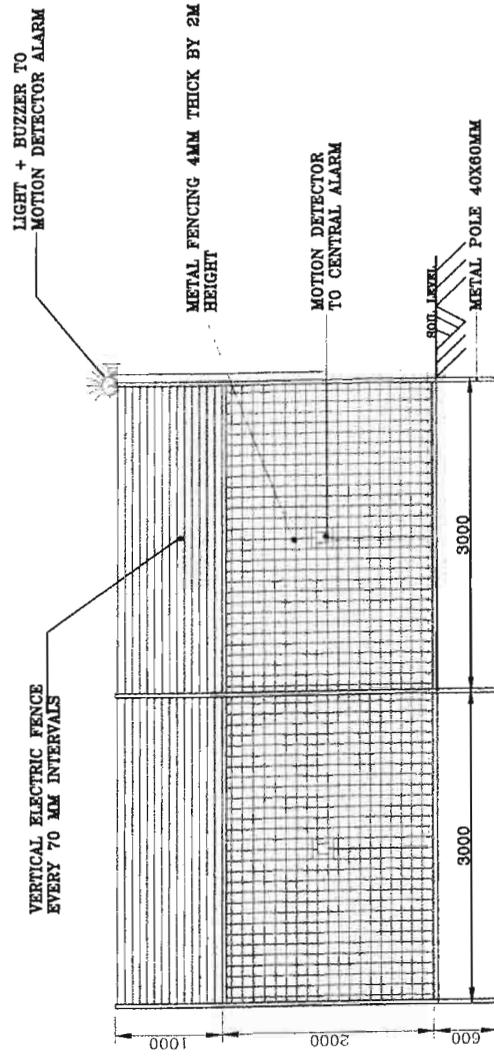
Hurricane Proof Cages

Cages especially designed & engineered to be Hurricane proof.



Double Electric Fence with motion sensors

SECURITY FENCING DETAILS FOR
BIOCULTURE PUERTO RICO

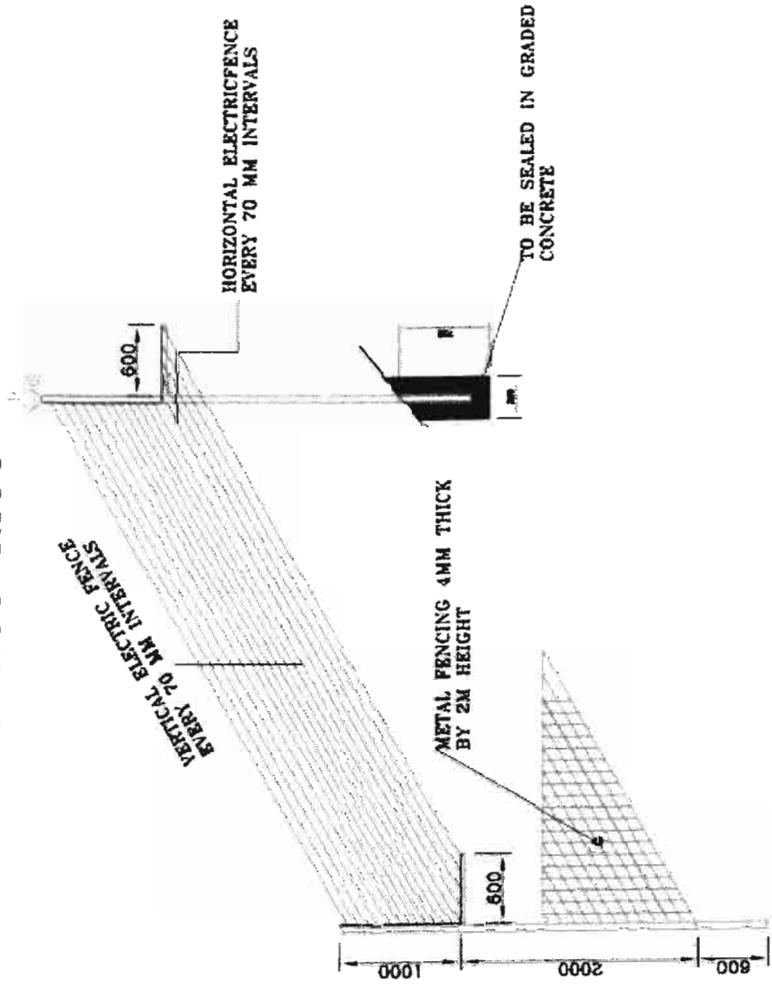


FRONT ELEVATION

NOTE:— ALL DIMENSIONS ARE IN MILLIMETRES

Double Electric Fence with motion sensors

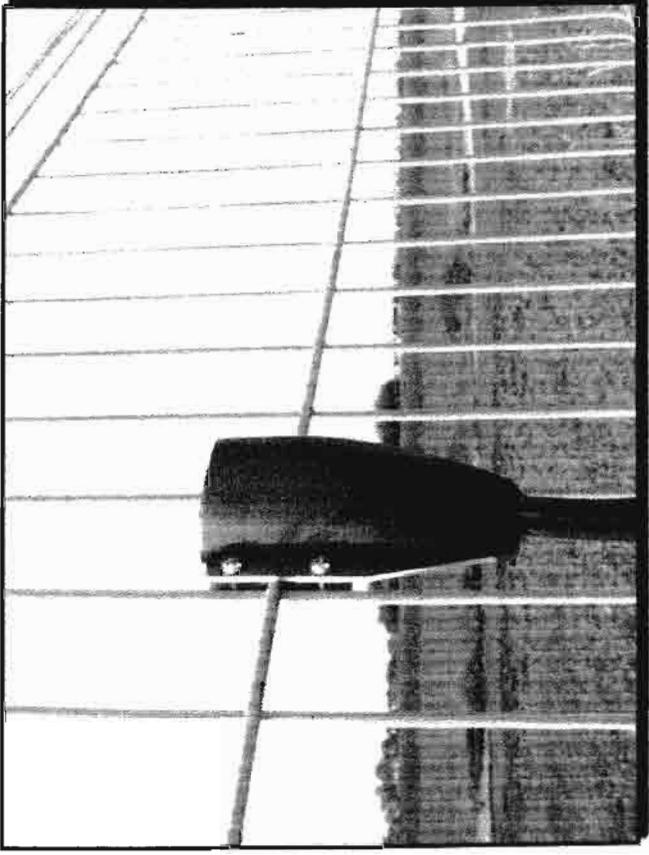
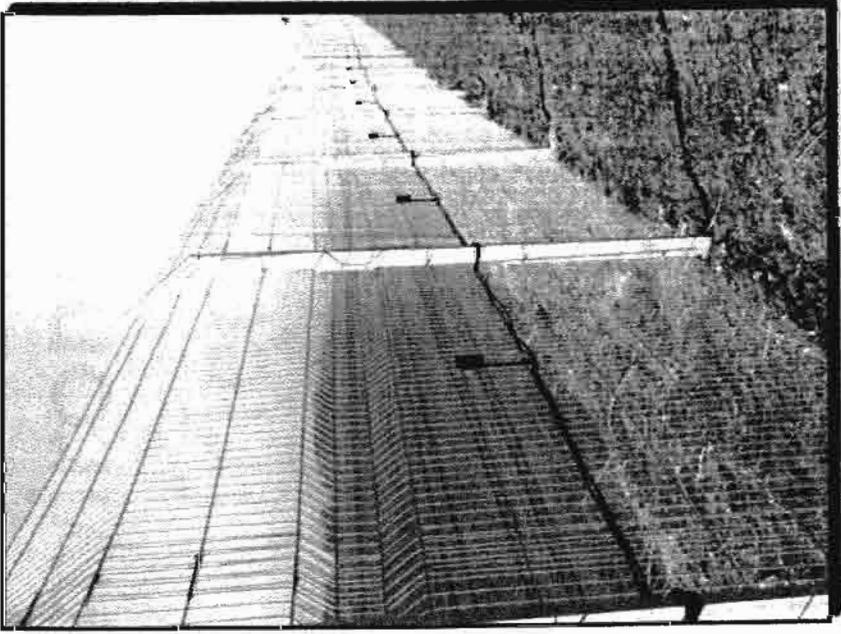
SECURITY FENCING DETAILS FOR
BIOCULTURE PUERTO RICO



SIDE ELEVATION SHOWING HORIZONTAL & VERTICAL
FENCING EVERY 70 MM INTERVALS

NOTE: - ALL DIMENSIONS ARE IN MILLIMETRES

Motion sensors



BIOCULTURE PR LTD PROCEDURE MANUAL

Section : QP 805-03	Issue No. : 2
Title : Disaster preparedness plan	Date of Issue : 20 August 2009
	Page : 1 of 3

1. Scope

The purpose of this procedure is to define how the Organization responds to a disaster in the event of its occurrence.

2. Associated documents

ISO 9001: 2000 Section 8.5
NRC Guide for the care and use of laboratory animals: Chapter 1
Quality Manual: QM108
Procedure Manual: QP805-01, QP805-02

3. **Distribution list:** **All Heads of Department**
Mechanical & Electrical Supervisor
Site Supervisors
Vet Staff
Technical Officer

4. Responsibilities

- .1 BCPR Management team is responsible for the effective implementation of this procedure.
- .2 All designated persons identified shall make themselves available as required.

5. Working protocol

6. Policies:

- .1 BCPR prepares an action plan in response to any disaster based on its specificities.

7. Definition of “disaster” within context of BCPR activities/operations:

Any situation which leads to the significant disruption of normal activities as a result of natural calamities or other acts is termed a disaster.

Compiled by:

Management Team

BIOCULTURE PR LTD PROCEDURE MANUAL

Section : QP 505-03	Issue No. : 2
Title : Disaster preparedness plan	Date of Issue : 20 August 2007
	Page : 2 of 3

8. Situations for a disaster

- .1 BCPR could face a disaster situation under any of the following conditions:
 - After a severe Tropical storm
 - Severe flood
 - Severe drought
 - Fire
 - Disease outbreak (human or animal)
 - Sabotage
 - Attack of BCPR premises by animal rights activists
 - Explosion on site
 - Epidemic in neighbouring human population
 - Complete loss of computer data
- .2 It is pointed out that the above situations will be deemed disasters based upon their severity and impact on BCPR operations.
- .3 The decision to classify any of the above situations as disaster is determined by BCMPR Management team.

9. General procedures

- .1 BCPR Management team meets urgently in disaster situation and prepares an action plan for response. The action plan is specific based on the nature/situation which caused the disaster and generally includes the following:
 - An assessment of the disaster and its impact on :
 - Humans
 - Animals
 - Infrastructure
 - Determination of the root cause of the disaster.
 - Establish list of actions and prioritize them.
 - Establish working group(s), identify leader(s) and define responsibilities with timeframes, if applicable.

BIOCULTURE PR LTD PROCEDURE MANUAL

Section : QP 505-03	Issue No. : 2
Title : Disaster preparedness plan	Date of Issue : 20 August 2007
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- Consider the need to inform/communicate to:
 - Police department
 - Health authorities
 - Fire services
 - Neighbourhood community representatives
 - Press releases/communiqués
 - Division of Veterinary Services
 - Customers
 - Major suppliers
 - Airlines
 - Natural Resources
 - Hospitals/Clinics
 - Vet Consultant(s)
 - Personnel
 - Any other stakeholder
 - Decide who will communicate and the extent of information to be communicated.
 - Consider the need to restrict access/movement within/between sites.
 - Identify institutions (local/overseas) to be contacted for technical/expert advice.
 - Determine whether additional assistance (e.g. police) would be required.
 - Forecast how much time would be needed to get BCPR back to normal and plan for essential activities for this period of time.
- .2 BCPR maintains a list of persons along with contact details for disaster response. This list is updated by the Confidential Secretary on a yearly basis.
- .3 The procedures for corrective and preventive actions (QP 805-01) and emergency situations (QP 805-02) are reviewed after any disaster.

10. Records

Record ref	Title
QR 85-09	Disaster response contact list
QR 85-10	Disaster Assessment Report

BIOCULTURE Puerto Rico PROCEDURE MANUAL

Section	: QP805-02	Issue No.	: 1
Title	: Emergency situations	Date of Issue	: 1 st Sept 2009
Compiled by	: Vice -President	Approved by	: President

1. **Scope**

The purpose of this procedure is to define the steps to be followed at BCPR in the event of an emergency.

4. **Responsibilities**

4.1 The Facility Manager is responsible for the effective implementation of this procedure.

5. **Working Protocol**

6. **Policies**

6.1. The Storm Emergency Team list should be available in each Security personnel Office.

7. **Procedures**

	Activities	Responsibilities
1.	Tropical Storm Watch Establish Storm emergency team for an annual basis for each site and designate team leaders.	Facility Manager
	Upon issue of storm warning by the Meteorological office, carefully study the trajectory forecast and its magnitude.	Facility Manager
	Site Supervisor to liaise with concerned dept and ensure all items in checklist have been thoroughly verified.	Site Supervisor
	Visit all sites and ensure the safety of equipment and installations.	Site Supervisor/ Maintenance Officer
	Upon Issue of Hurricane Watch (refer to Annex 1): All company vehicles users to fill up their respective vehicle.	All company vehicles users
	For tunnel cages, close access to tunnels depending on assessment of weather conditions.	Site Supervisor
	Upon Issue of Hurricane warning: If early during the day, immediately upon issue of warning (refer to Annex 1), ensure all animals are fed with their normal food ration. Pellets and bread are also provided weather permits. If heavy rains prevail, a double fresh food ration is given in lieu of pellets.	Food Storekeeper
	All personnel go back home except those in Emergency Teams unless otherwise decided by Management based upon hurricane magnitude or trajectory forecast.	Site Supervisor

	Activities	Responsibilities
	<p>Note: While doing the following, the team leader shall make proof of good judgment in order not to compromise safety of team members. Emergency team to make hourly rounds of site in groups and:</p> <ul style="list-style-type: none"> a. Check any major damage to cages due to fallen trees or branches. b. Stop the generator for one hour after each 3 hours running. Clear drains in case they are blocked. c. Transfer any sick/injured animals to appropriate indoor buildings. d. Attend to provisional repairs where possible to buildings damaged (e.g. seal broken windows). 	Emergency team
	<p>After the Hurricane:</p> <ul style="list-style-type: none"> a. Make preliminary assessment of damage sustained. b. Attend to urgent actions required if possible. c. Feed animals with their normal ration (pellets can be given if weather permits otherwise double fresh food ration given). d. Report salient issues to personnel upon their arrival. e. Emergency teams leave the sites. f. Vet staff to conduct thorough verification and report their findings in cyclone report. g. Ensure availability of water for animals in case there is no town water supply. 	<p>Each Head of Department Food Storekeeper</p> <p>Vet Staff</p>
	<p>Hurricane Report</p> <ul style="list-style-type: none"> a. Emergency team leader to complete Hurricane Report and submit to Facility Manager. b. Maintenance officer to conduct survey on all sites and prepare the Infrastructure Damage Report within 24 hours of lifting of hurricane warning. 	<p>Emergency Team Leader</p> <p>Maintenance officer</p>
	<p>Both Hurricane Report and Infrastructure Damage Report are discussed by Management. The effectiveness of this procedure is assessed and actions deemed necessary are initiated.</p>	Facility Manager/ Management
	<p>Actions taken with respect to hurricane reports are discussed during Ethical Review Committee meetings if relevant.</p>	Facility Manager

Records and Specification Table

Record ref	Title
QR5-05	Minutes of Meeting
QR62-07	Training Record Sheet
QR64-19	List of Fire Extinguishers
QR85-05	Hurricane Checklist
QR85-06	Hurricane Report
QR85-07	Infrastructure Damage Report (Hurricane)
TAB8-1	Hurricane Emergency Team

Animal welfare issues

1. After any emergency situation, priority shall be given to depicting/attending sick/wounded animals.
2. Ensure all enrichment devices have been removed (based on intensity of cyclone).
3. Ensure availability of food and water.
4. Priority shall be given to all repairs related to cage infrastructure.

Annex 1

- **TROPICAL STORM WATCH**
A tropical storm watch is issued when tropical storm conditions, including winds from 39 to 73 miles per hour (mph), pose a possible threat to a specified coastal area within 36 hours.
- **TROPICAL STORM WARNING**
A tropical storm warning is issued when tropical storm conditions, including winds from 39 to 73 mph, are expected in a specified coastal area within 24 hours or less.
- **HURRICANE WATCH**
A hurricane watch is issued for a specified coastal area for which a hurricane or a hurricane-related hazard is a possible threat within 36 hours.
- **HURRICANE WARNING**
A hurricane warning is issued when a hurricane with sustained winds of 74 mph or higher is expected in a specified coastal area in 24 hours or less. A hurricane warning can remain in effect when dangerously high water or a combination of dangerously high water and exceptionally high waves continues, even though the winds may have subsided below hurricane intensity.

. **Termination:** Issued when there is no longer any appreciable danger of gusts exceeding 120 km per hour.

**6 Hour
Forecast Cycle**
When a storm threatens
the following occurs

0:00 A new hurricane forecast cycle begins.

0:45 Receive the location of the center of the hurricane.

1:00 Initialize or start the **hurricane models** with the storm's location and intensity

1:20 Receive model guidance and prepare a new hurricane forecast.

2:00 **Coordinate** with National Weather Service and Dept. of Defense.

3:00 Issue the full **hurricane advisory package**.

ISSUANCE TIME:
5am EDT (4 CDT)
11am EDT (10 CDT)
5pm EDT (4 CDT)
11pm EDT (10 CDT)

3:15 Participate in the Federal Emergency Management Agency (FEMA) conference call with the affected states.

6:00 A new hurricane forecast cycle begins.

****** When a Watch or a Warning is issued, intermediate advisories are initiated.

[MORE INFO ON FORECAST PRODUCTS](#)

**BIOCULTURE PR LTD
PROCEDURE MANUAL**

Section : QP805-02	Issue No. : 5
Title : Emergency situations - Tropical storm	Date of Issue : 12 November 2008
Compiled by : Operations Manager	Approved by : General Manager

1. **Scope**

The purpose of this procedure is to define the steps to be followed at BCPR in the event of an emergency.

4. **Responsibilities**

4.1 The Operations Manager is responsible for the effective implementation of this procedure.

4.2 All designated persons identified to deal with an emergency situation shall make themselves available in case of need.

5. **Working Protocol**

6. **Policies**

6.1. The Tropical storm Emergency Team list (**TAB8-1**), should be available in each Security personnel Office.

7. **Procedures**

	Activities	Responsibilities	Records
1.	Tropical storm Establish Tropical storm emergency team for an annual basis for each site and designate team leaders. Operations Manager to submit Tropical storm checklist to Site Supervisor.	Operations Manager/ Site Supervisor / Quality Assurance Officer	TAB8-1
	Upon issue of Class I Tropical storm warning by the Meteorological office (refer to Annex 1), carefully study the trajectory forecast and its magnitude.	General Manager/ Operations Manager	--
	Site Supervisor to liaise with concerned dept. and ensure all items in checklist have been thoroughly verified.	Site Supervisor	QR85-05
	Visit all sites and ensure the safety of equipment and installations.	Mechanical & Electrical Manager Technical Officers	--
	Upon Issue of Class II warning (refer to Annex 1): All company vehicles users to fill up their respective vehicle.	All company vehicles users	--
	For tunnel cages, close access to tunnels depending on assessment of weather conditions.	Site Supervisor	--
	Upon Issue of Class III warning: If early during the day, immediately upon issue of Class III warning (refer to Annex 1), ensure all animals are fed with their normal food ration. Pellets and bread are also provided if weather permits. If heavy rains prevail, a double fresh food ration is given in lieu of pellets.	Food Storekeeper	--
	All personnel go back home except those in Emergency Teams unless otherwise decided by Management based upon Tropical storm magnitude or trajectory forecast.	Site Supervisor	--

Activities	Responsibilities	Records
<p>Note: While doing the following, the team leader shall make proof of good judgment in order not to compromise safety of team members. Emergency team to make hourly rounds of site in groups and:</p> <ol style="list-style-type: none"> Check any major damage to cages due to fallen trees or branches. Stop the generator for one hour after each 3 hours running. Clear drains in case they are blocked. Transfer animals requiring intensive (e.g. incubator and heater) care to where electricity supply is continuous. Transfer any sick or injured animals to internal building. Attend to provisional repairs where possible to buildings damaged (e.g. seal broken windows). 	Emergency team	--
<p>After the Tropical storm:</p> <ol style="list-style-type: none"> Make preliminary assessment of damage sustained. Attend to urgent actions required if possible. Feed animals with their normal ration (pellets can be given if weather permits otherwise double fresh food ration given). Report salient issues to personnel upon their arrival. Emergency teams leave the sites. Vet staff to conduct thorough verification and report their findings in Tropical storm report. Ensure availability of water for animals in case there is no town water supply. 	<p>Each Head of Department Food Storekeeper</p> <p>Vet Staff</p>	QR85-06
<p>Tropical storm Report</p> <ol style="list-style-type: none"> Emergency team leader to complete Tropical storme Report and submit to Operations Manager. Technical officer to conduct survey on all sites and prepare the Infrastructure Damage Report within 24 hours of lifting of Tropical storm warning. 	<p>Emergency Team Leader</p> <p>Technical officer</p>	<p>QR85-06</p> <p>QR85-07</p>
<p>Both Tropical storm report and Infrastructure Damage Report are discussed by Management. The effectiveness of this procedure is assessed and actions deemed necessary are initiated.</p>	General Manager/ Operations Manager/ Accounts & Planning Manager	QR85-06 QR85-07
<p>Actions taken with respect to Tropical storm reports are discussed during Ethical Review Committee meetings if relevant.</p>	General Manager	QR5-05

	Activities	Responsibilities	Records
2.	Floods In case of heavy rains, likely to result in flooding of premises, Site Supervisor to ensure: a. All water exits are cleared and free from any material which might prevent the flow of water b. Create temporary/additional water exits if necessary and direct water to the stream or furthest possible location from premises. c. Regularly inspect water exits to remove any accumulated mud / materials, which may obstruct the water flow. d. After heavy rains have stopped, discharge any accumulated water.	Site Supervisor	--
3.	Fire Ensure adequacy of fire extinguishers and their locations at all sites Ensure that each site has at least 2 persons trained in the use of fire extinguishers.	Security Manager/ Health & Safety Officer	QR64-19 QR62-07
	In the event of fire outbreak, use water or fire extinguishers accordingly.	All persons	--
	In case it is found that fire is not within control, immediately phone the Fire Brigade. Inform Operations Manager/General Manager. Security Officer/ Site Supervisor to sound alarm.	Site Supervisor Operations Manager Security Officer	--
	Personnel must not endanger their lives while putting out fire.	--	--
	Collaborate/ Provide assistance to Fire Brigade. Remove obstruction to facilitate access to fire outbreak.	All persons	--

8. In case of a disease outbreak, the Vet Manager shall immediately inform the General Manager. An urgent management meeting is convened to decide upon lines of action on a case to case basis.
9. All Site Supervisors are members of BCPR Health and Safety Committee.

Records and Specification Table

Record ref	Title
QR5-05	Minutes of Meeting
QR62-07	Training Record Sheet
QR64-19	List of Fire Extinguishers
QR85-05	Tropical storm Checklist
QR85-06	Tropical storm Report
QR85-07	Infrastructure Damage Report (Tropical storme)
TAB8-1	Tropical storm Emergency Team

Animal welfare issues

1. After any emergency situation, priority shall be given to depicting/attending sick/wounded animals.
2. Ensure all enrichment devices have been removed (based on intensity of Tropical storm).
3. Ensure availability of food and water.
4. Priority shall be given to all repairs related to cage infrastructure.

Annex 1

THE TROPICAL STORM WARNING SYSTEM:

Class I

Issued 36 to 48 hours before the advent of Tropical storm conditions.

Class II:

Issued so as to allow, as far as practicable, 12 hours of daylight before the occurrence of gusts of 120 kilometers (km) per hour.

Class III:

Issued so as to allow, as far as practicable, 6 hours of daylight before the occurrence of gusts of 120 kilometers (km) per hour.

Class IV:

Issued when gusts of 120 km per hour have been recorded and are expected to continue to occur.

Termination: Issued when there is no longer any appreciable danger of gusts exceeding 120 km per hour.

CLASSIFICATION OF TROPICAL STORM:

Zone of disturbed weather (or tropical disturbance):

An area of low pressure relative to the surrounding region; the associated cloud masses are usually not well-organized.

Tropical depression:

A non-frontal synoptic scale low-pressure system originating over tropical waters with enhanced convection and/or some indications of Tropical storm wind circulation. Winds circulate clockwise around low-pressure and Tropical storm systems in the southern hemisphere. Gusts associated with tropical depression are generally less than 89 kilometers (km) per hour.

Moderate tropical storm:

A non-frontal synoptic scale low-pressure system originating over tropical waters with organized convection and definite Tropical storm wind circulation. Estimated gusts associated with moderate tropical storms range from 89 to 124 km per hour.

Severe tropical storm:

A tropical storm in which the estimated wind gusts range from 125 to 165 km per hour.

Tropical Tropical storm:

A tropical storm in which the estimated wind gusts range from 166 to 233 km per hour.

Intense tropical Tropical storm:

A tropical storm in which the estimated wind gusts range from 234 to 299 km per hour.

Very intense tropical Tropical storm:

A tropical storm in which estimated gusts exceed 300 km per hour.



GOBIERNO DE PUERTO RICO
Departamento de Recursos Naturales y Ambientales

PERMISO

Autorizado: Bioculture PR, Inc.
326 Rey Francisco,
Guaynabo, PR 00969
(787) 361-8717

Número DRNA: 2010-IC-025
O-VS-PSV15-SJ-00416-0502-2010

Expira: 26 de mayo de 2015

Tipo de Permiso: Investigación Científica

Lugar donde se autoriza a llevar a cabo la actividad objeto de este Permiso:

Carr. 712 Km. 14.9, Bo. Pozo Hondo
Guayama, PR 00784

1. Condiciones y autorización:

- 1.1. La validez de este Permiso depende de que las actividades aquí autorizadas se lleven a cabo de acuerdo a las leyes y reglamentos estatales y federales aplicables y de que se cumpla con las condiciones aquí estipuladas.
- 1.2. Este Permiso es intransferible y sujeto a revisión o cancelación si las circunstancias, a juicio del Departamento de Recursos Naturales y Ambientales (DRNA), así lo ameritan.
- 1.3. Este Permiso no será válido sin los permisos federales y locales correspondientes de éstos ser requeridos.
- 1.4. Este Permiso deberá ser portado por su tenedor en todo momento durante su uso.
- 1.5. Se autoriza a Bioculture PR, Inc. y personal autorizado, a importar y exportar para fines científicos, hasta un máximo de cuatro mil quinientos (4,500) individuos de la especie *Macaca fascicularis*, durante el término de vigencia de este Permiso, según se desglosa a continuación:
 - 1.5.1. Setecientos cincuenta (750) animales durante el **primer año de vigencia** de este Permiso.
 - 1.5.2. Mil (1,000) animales durante el **segundo año de vigencia** de este Permiso.
 - 1.5.3. Mil (1,000) animales durante el **tercer año de vigencia** de este Permiso.
 - 1.5.4. Mil (1,000) animales durante el **cuarto año de vigencia** de este Permiso.
 - 1.5.5. Setecientos cincuenta (750) animales durante el **quinto año de vigencia** de este Permiso.
- 1.6. Bioculture PR, Inc. tendrá que someter evidencia de que los primates exportados serán utilizados exclusivamente para realizar investigación científica. Se aceptará como evidencia documentación escrita sobre la transacción de compraventa o cesión de animales entre Bioculture PR, Inc. y la compañía o institución recipiente. Igualmente, Bioculture PR, Inc. deberá demostrar por escrito que dicha compañía o institución se dedica a la investigación científica antes de solicitar la autorización de exportación por parte del DRNA. La información incluirá pero no se limitará a informar el nombre de la compañía, dirección en la red cibernética y nombre de persona contacto.

(X)

1.7. Este Permiso se concede sujeto a las siguientes condiciones:

- 1.7.1. Será ilegal vender, ceder o transferir los animales en cuestión a otra persona o entidad, dentro del territorio de Puerto Rico, excepto a las entidades académicas de educación universitaria dedicadas a investigación científica en Puerto Rico. De Bioculture PR, Inc. realizar cualquier venta, cesión o transferencia a cualquier entidad autorizada por Ley para ello, deberá notificarlo al DRNA dentro de un término de treinta (30) días, a partir de la fecha en que se realice la transacción. La notificación deberá incluir el número de primates que hayan sido vendidos, cedidos o transferidos.
- 1.7.2. Los animales no podrán ser liberados al medioambiente en Puerto Rico bajo ninguna circunstancia.
- 1.7.3. Deberá notificar al DRNA, vía telefónica al (787) 724-5700, dentro de un término no mayor de veinticuatro (24) horas, en la eventualidad de pérdida de los animales objeto de este Permiso. El incumplimiento de esta disposición podrá conllevar la revocación inmediata de este Permiso.
- 1.7.4. Deberá presentar certificado veterinario de cada uno de los sujetos de la especie en cuestión.
- 1.7.5. Deberá notificar al Cuerpo de Vigilantes con cuarenta y ocho (48) horas de anticipación al embarque, el número de vuelo, línea aérea y hora exacta en que los animales serán importados o exportados, llamando al (787) 253-3857 ó 724-5700.
- 1.7.6. No se inspeccionarán embarques sábados, domingos y días feriados. Todo embarque que llegue después de las 10:00 p.m., en días laborables será inspeccionado el próximo día laborable.
- 1.7.7. Deberá solicitar la renovación al menos con noventa (90) días, previos a la fecha de expiración del Permiso.
- 1.7.8. La instalación contará con máxima seguridad. Lo anterior incluye, pero no se limita, a lo siguiente: doble verja alrededor del perímetro de las jaulas, la verja interior del perímetro deberá estar electrificada, guardias de seguridad las veinticuatro (24) horas, alarmas contra escape y detectores de movimiento en la verja del perímetro interior, conectado al sistema de alarmas.
- 1.7.9. Se deberá rotular las áreas externas a la Finca con letreros de aviso a la ciudadanía sobre la peligrosidad de acercarse a las verjas antes mencionadas.
- 1.7.10. Bioculture PR, Inc. tendrá responsabilidad exclusiva sobre cualquier daño causado durante su operación, como parte de sus operaciones y dentro de su propiedad. Será requisito que Bioculture PR, Inc. obtenga un seguro de responsabilidad pública, por no menos de un millón de dólares (\$1,000,000.00) con un primer endoso a favor del Gobierno de Puerto Rico, un segundo endoso a favor del DRNA y del Departamento de Agricultura. Dicha póliza deberá estar al día durante la vigencia del Permiso y no podrá ser modificada sin la previa notificación y consentimiento del DRNA.

DeD

- 1.7.11. Bioculture PR, Inc. deberá obtener y presentar una fianza de cumplimiento a favor del DRNA, según se desglosa a continuación:
- 1.7.11.1. Seiscientos mil dólares (\$600,000) para el **primer año de vigencia** de este Permiso.
 - 1.7.11.2. Un millón, cuatrocientos mil dólares (\$1,400,000) para el **segundo año de vigencia** de este Permiso.
 - 1.7.11.3. Dos millones, doscientos mil dólares (\$2,200,000) para el **tercer año de vigencia** de este Permiso.
 - 1.7.11.4. Tres millones de dólares (\$3,000,000) para el **cuarto año de vigencia** de este Permiso.
 - 1.7.11.5. Tres millones seiscientos mil dólares (\$3,600,000) para el **quinto año de vigencia** de este Permiso.
- 1.7.12. La fianza de cumplimiento deberá estar vigente y al día durante la vigencia de este Permiso, según se desglosa en el acápite que antecede. La Fianza de cumplimiento no podrá ser modificada, sin la previa notificación y consentimiento expreso del DRNA. La fianza antes mencionada será ejecutable bajo las siguientes circunstancias: **(1)** abandono de la operación en Puerto Rico por parte de Bioculture PR, Inc.; **(2)** escape de animales en donde Bioculture PR, Inc. no pueda capturarlos en un periodo de cuatro (4) horas y el Departamento tenga que realizar su captura. El costo de la captura se realizará con cargo a la fianza de cumplimiento; **(3)** escape de animales como consecuencia de desastres naturales o causa mayor; **(4)** quiebra y liquidación de Bioculture PR, Inc.; **(5)** daños causados por escapes de animales debido a negligencia atribuible a Bioculture PR, Inc. en sus operaciones.
- 1.7.13. Bioculture PR, Inc. contará con un término de treinta (30) días para presentar evidencia al DRNA de la obtención de la póliza de responsabilidad pública y la fianza de cumplimiento, según establecida para **cada año de vigencia** de este Permiso.
- 1.7.14. El DRNA por conducto de la Unidad de Vida Silvestre del Cuerpo de Vigilantes, podrá en cualquier momento y sin previo aviso, entrar a las instalaciones de Bioculture PR, Inc., para inspeccionar que las condiciones de este Permiso se estén cumpliendo. Mediante la firma de este documento Bioculture PR, Inc. renuncia expresamente a cualquier reclamo de derecho a la intimidad o de registros irrazonables, relacionados con las inspecciones que puedan llevarse a cabo por el DRNA.
- 1.7.15. En caso de incumplimiento con cualquiera de las condiciones de este Permiso, el DRNA podrá revocar el mismo en cualquier momento.
- 1.7.16. En caso de que se escape cualquier primate por la razón que fuere, Bioculture PR, Inc. será responsable del rastreo y captura del mismo. Si dentro de las cuatro (4) horas del escape, Bioculture PR, Inc. no ha podido atrapar el primate, tendrá la obligación de notificar este hecho al DRNA, quién se encargará de la captura del mismo con cargo a la fianza de cumplimiento. De Bioculture PR, Inc. incumplir con esta disposición, la fianza será ejecutada y el Permiso será revocado inmediatamente.



- 1.7.17. Este Permiso tendrá una vigencia de cinco (5) años, sujeto a la presentación de copia certificada de la póliza de responsabilidad pública y la fianza de cumplimiento, según desglosada en el Inciso 1.7.11.
- 1.7.18. Bioculture PR, Inc. será responsable de someter copia certificada de las primas de fianza de cumplimiento, según las cantidades establecidas en el Inciso 1.7.11, con treinta días de anticipación a la fecha en que entre en vigor el segundo, tercero, cuarto y quinto año de vigencia del Permiso. El incumplimiento con esta disposición dará lugar a la revocación de este Permiso y la ejecución de la fianza de cumplimiento.
- 1.7.19. Este Permiso será firmado y aceptado por Bioculture PR, Inc. o su Representante Autorizado.
- 1.7.20. Este Permiso tendrá vigencia y operará en pleno vigor cuando **Bioculture PR, Inc. haga entrega al DRNA de copia certificada de las primas de seguro de responsabilidad pública y de la fianza de cumplimiento requerida para cada año de vigencia de este Permiso.**

2. **Requisitos de Informe:** El Peticionario deberá rendir **UN INFORME** detallado de las actividades realizadas al amparo de este Permiso treinta (30) días antes de la fecha de expiración, disponiéndose que transcurrido el término sin haber presentado el Informe, el Departamento podrá incautar y disponer de cualquier especie autorizada en el Permiso, no renovar el Permiso o tomar acciones legales y administrativas que en derecho procedan.

Expedido por:



Daniel J. Galán Kercadó
Secretario

Fecha:

26 de mayo de 2010

Yo, _____ libre y voluntariamente, certifico que me encuentro autorizado a comparecer a nombre y en representación de Bioculture PR, Inc., que he leído todos y cada uno de los términos y condiciones contenidos en presente Permiso y que acepto cumplir con los mismos, según han sido transcritos en este documento.

Representante Autorizado de
Bioculture PR, Inc.
Firma

Fecha

Secretariat number

ESTADO LIBRE ASOCIADO DE PUERTO RICO
DEPARTAMENTO DE RECURSOS NATURALES Y
AMBIENTALES
P.O. BOX 366147
SAN JUAN PR 00936

APPLICATION FOR SCIENTIFIC PURPOSES

Type of application:

Vulnerable species or species in danger Wild life not designated
 New Renovation Last permit number: _____
 Amendment Permit Number: _____

1. Name: BUSHMITZ MOSHE

2. Social Security: _____

3. Postal Address: 326 Rei Francisco , Guaynabo , Puerto Rico 00969

4. Home Address (Residential Country) : 326 Rei Francisco , Guaynabo , Puerto Rico 00969

5. Home Phone Number: 787 3618717

6. Work Phone Number: 787 3618717

7. Address in Puerto Rico: Pueblito Carmen, Box HC02-4250 GUAYAMA PR 00784

8. Institution, public agency or private entity responsible:

A. Name: Bioculture PR inc.

B. Address: Pueblito Carmen, Box HC02-4250 GUAYAMA PR 00784_

C. Thesis counselor, Supervisor, President or any other authorized representative that makes responsible:

1. Name: Prof. Bushmitz Moshe
2. Title: Vice president
3. Phone: 787-3618717
4. Signature:

9. Requested Activity: _____ Collection _____ Capture _____ Other Explain:

importation , breeding exportation and holding for scientific purposes (biomedical research)

10. Purpose of the requested activity: _____ Educational Scientific

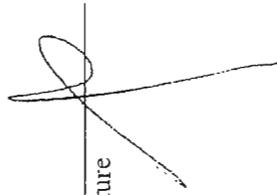
11. Place where the activity will take place: **Physical Address :**
Carr. 712 KM 14.9 BO.Pozo Hondo GUAYAMA PR 00784

12. Have you ever been intervened, convicted or fined for violation of laws or rules, state of federal, related to wild life:

No Yes If so, indicate date and type of fine or conviction:

I certify that all the information stated above is true.

Signature



Date

Requirements:

- A. Submit permit application with at least ninety (90) work days of anteriority to the date that you are applying for permit at the Secretary's office or at the Regional Department Office.
- B. Certified Check, receipt of payment sent by a Department collector or money order to the amount of \$25.00, addressed to the secretary of housing.
- C. Work proposal that includes the following information:
 - Scientific and common name of the interested species.
 - Quantity, age and sex of the unit species that you wish to collect or manipulate.
 - Goals, objectives and expected benefits as result of the investigation.
 - Detailed methodology and duration of the project.
 - Possible impacts.
 - Final Disposition of the collected individuals.
- D. Curriculum Vitae of the Principal investigator.
 - In case it is a student, recommendation from his/her graduating committee or counselor.
- E. In case of renovation, submit detailed report of the activities performed, at least, 30 days before the culmination of the activity approved on the last permit.

Secretariat Office use only

Signature of the civil servant who received the application

Luz I. Toires'

From: Irmaris Vicenty Berríos
Sent: Friday, February 05, 2010 9:58 AM
To: Luz I. Torres
Subject: FW: Bioculture PR - Investigación Científica

Attachments: Importation Permit application Scientific.pdf

From: Federico Ching
[mailto:drfching14@yahoo.com]
Sent: Friday, February 05, 2010 5:58 AM
To: Irmaris Vicenty Berríos
Cc: Victor Merced PRIDCO; Emil Lawyer
Subject: Bioculture PR - Investigación Científica

Lucy:

Por favor imprime este documento.
Paso a recogerlo en un rato. Gracias.

*LCD.A. IRMARIS VICENTY
BERRÍOS*

AYUDANTE ESPECIAL

Oficina del Secretario

Tel. (787) 999-2200 Ext.2167

www.ivicenty@drna.gobierno.pr

AVISO DE CONFIDENCIALIDAD: Este correo electrónico y/o el material adjunto es para uso exclusivo de la persona o entidad a la que expresamente se le ha enviado, y puede contener información confidencial o material privilegiado. Si usted no es el destinatario legítimo del mismo, por favor repórtelo inmediatamente al remitente del correo y bórralo. Cualquier revisión, retransmisión, difusión o cualquier otro uso de este correo, por personas o entidades distintas a las del destinatario legítimo, queda expresamente prohibido. Este correo electrónico no pretende ni debe ser considerado como constitutivo de ninguna relación legal, contractual o de otra índole.

Buenos dias Lcda. Irmaris,

Le adjunto la documentacion requerida en el email enviado al Lcdo Emil Rodriguez Escudero. Este documento fue incluido en la solicitud inicial enviada a su institucion. Estare comunicandome con Ud. en la manana de hoy viernes 5 de febrero para verificar que recibio la misma y al mismo tiempo saber si se necesita algo mas a parte del cheque certificado de \$25.00 para continuar con el proceso del permiso de importacion.

Saludos,

Dr. Federico S. Ching
Gerente General
Bioculture PR
787-436-6603

From: "Irmaris Vicenty Berríos"
<ivicenty@drna.gobierno.pr>
Date: February 3, 2010 4:25:09
PM GMT-04:00
To: <emil@mlrelaw.com>
Cc: Daniel Galán Kercadó
<dgalan@drna.gobierno.pr>
**Subject: Presentación de
Solicitud de Permiso**

Saludos Lcdo. Rodriguez:

Por instrucciones recibidas, remito a su atención solicitud de permiso para investigación científica. Para dar comienzo al trámite administrativo para la aprobación del permiso de Bioculture es necesario que la solicitud sea debidamente cumplimentada y presentada ante la Secretaría de nuestro Departamento. La solicitud deberá estar acompañada de un cheque certificado o giro por la

cantidad de \$25.00 a favor del Secretario de Hacienda. De tener cualquier duda o pregunta te puedes comunicar conmigo a tu mejor conveniencia.

*LCCDA. IRMARIS
VICENTY BERRÍOS*

AYUDANTE ESPECIAL

Oficina del Secretario

Tel. (787) 999-2200
Ext.2167

www.ivicenty@drna.gobierno.pr



Estado Libre Asociado de Puerto Rico
DEPARTAMENTO DE RECURSOS NATURALES
Y AMBIENTALES

HOJA DE TRÁMITE

10 mar 10

FECHA 22 de febrero de 2010

INICIALES

1ro HON. DANIEL J. GALAN KERCADO
SECRETARIO

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2010 FEB 22 11 4 19
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DE: OFICINA DE SECRETARIA

BIOCULTURE PUERTO RICO, INC (BIOCULTURE) ANIMAL
IMPORTATION PERMIT

CORRESPONDENCIA DE:
FECHA: 19-FEBRERO-10

MOSHE BUSHMITZ - BIOCULTURE PR, INC
A LA MANO

- | | |
|---|--|
| <input type="checkbox"/> Contestar P/F | <input type="checkbox"/> Someter recomendaciones al Secretario |
| <input type="checkbox"/> Contestar directamente | <input type="checkbox"/> Investigar e informar |
| <input type="checkbox"/> Acusar recibo e informar acción a tomar | <input type="checkbox"/> Someter Informe |
| <input checked="" type="checkbox"/> Acción pertinente | <input type="checkbox"/> Enterarse y devolver |
| <input type="checkbox"/> Discutir conmigo | <input type="checkbox"/> Mantener al Secretario informado |
| <input type="checkbox"/> Discutir con el Secretario de estimarlo pertinente | <input type="checkbox"/> Devolver |

Observaciones:

OFICINA DE RECURSOS NATURALES
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23
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BIOCULTURE
CARING FOR LIFE

PA
AES - 2/19/10
AMG
2010 FEB 22 11 4 19

BIOCULTURE (Puerto Rico) Inc.
Pueblito Carmen, Box HC02-4250
GUAYAMA PR 00784
Tel: +1(787) 200 4572
Email: info@bcmpr.com

February 19, 2010

Hon. Daniel J. Galán Kercadó
Secretary
Department of Natural Resources
P O Box 366147
San Juan Puerto Rico 00936



Re: Bioculture Puerto Rico, Inc. (Bioculture) Animal Importation Permit

Esteemed Secretary:

On December 9, 2009, we had a meeting with you and members of the Puerto Rico Industrial Development Corporation (PRIDCO), with the purpose of submitting Bioculture's application for an animal importation permit. The complete application was personally given to you based on your representations that the same was going to be filed in the Department of Natural Resources and Environment on the same day. Resting on what you stated we are assuming that the application was filed as promised. We have not received any formal communication from the Department with regard to the sufficiency of the application or the need to further supply any information necessary for its consideration.

The pertinent regulation provides that said permit application is to be acted upon within 60 days of its filing. Based on the December 9 filing date, I informed Bioculture Mauritius, Ltd.'s Board of Directors as well as some of our clients that closely follow the development of the project in Puerto Rico and that are seriously considering investing in medical research facilities in the Island subject to the availability of our product, that the importation permit would be decided during the first half of February.

As of today, and having the 60-day term expired, we have not received a formal response from the Department regarding our application. In the meantime, we continue with the construction of the facilities for the breeding of primates for scientific research, as approved by the Permits and Regulations Administration

and not opposed by your Department. As a matter of fact, Bioculture has invested over 2 million dollars in the land acquisition, the construction of the facilities, including, payroll for dozens of Puertorrican employees, and professional services, among others. Our investment is the result of the trust deposited on the government's support of the project, manifested in the representations made to us and as evidenced by the issuance of the construction permit and its defense in the ensuing litigation, a matter for which we are grateful. There should be no doubt that Bioculture has a proprietary interest in the animal breeding project as designed and its construction permit authorized. The animal importation permit is a *sine qua non* element of the project. Without said permit, all other permits and our investment would be an exercise in futility. The government cannot, on one hand, permit the construction of an animal breeding facility, the production of which will be for scientific research, and on the other hand, do not permit the importation of the animals therefore. That would certainly be, in our opinion, a deprivation of property without due process.

Furthermore, the delay in the consideration of our application for the importation permit is causing us substantial damages. Not only to Bioculture, but also to the many employees that are presently without workshop due to the absence of what is currently the most important step in the development of Bioculture's Puerto Rico operation, to wit, the animal importation permit. The creation of jobs and the growth of the economy is the primary public policy of the Government of Puerto Rico. Bioculture is presently unable to contribute to the creation of permanent jobs in an economically depressed area due to the uncertainty of timely having the requested permit. This, of course, also bars the establishment in Puerto Rico of important research facilities with the probable employment of thousands of well paid workers, with the additional known consequences in the Island's economy and scientific development.

Article 8.01 of Regulation 6765 is clear. The permit in question is required to all persons that are dedicated, totally or partially, to the importation of exotic animals for the purpose of scientific research (con fines de investigación científica). **It is not a condition of the permit that the importer be the scientific researcher or that it performs scientific research on the animals.** What is required is that the animal be imported for scientific research.

It is known by all concerned, from the very beginning, that Bioculture is an animal breeding enterprise and its product is sold only and exclusively for scientific uses. All the permits and endorsements that have been obtained as of this date are based on that fact. [See, in addition, our permit application, and the

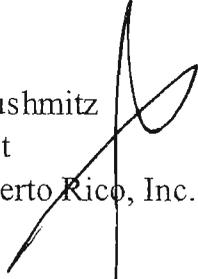
attached sworn statement]. Furthermore, nowhere in Regulation 6765 is stated that the importer has to do research on the imported animal. That requirement is for the end use of the animal.

Consequently, and in view of the clear regulatory provisions on the matter, it follows that our application be approved. Of course, the Department may impose the reasonable conditions and restrictions that it may deem necessary within the framework of Regulation 6765.

The swift solution of this permit application will be consistent with the development of the Bioisland concept in order to promote the creation of new permanent jobs in Puerto Rico.

Respectfully,

Dr. Moshe Bushmitz
Vice-President
Bioculture Puerto Rico, Inc.



C.c. – Owen Griffiths, President Bioculture Mauritius, Ltd.
Javier Vázquez, Director PRIDCO

SWORN STATEMENT REGARDING THE APPLICATION FOR ANIMAL IMPORTATION
PERMIT OF BIOCULTURE PUERTO RICO, INC. PRESENTED AT THE
DEPARTMENT OF NATURAL AND ENVIRONMENTAL RESOURCES OF PUERTO RICO

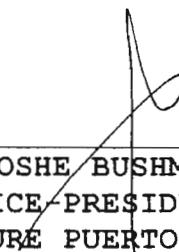
I, **MARK MOSHE BUSHMITZ**, of legal age, married, Vice -
President of Bioculture Puerto Rico, Inc., and with residence in
the Country of Israel, **UNDER SOLEMN OATH**, hereby declare:

1. My name and personal circumstances are as above stated.
2. I am the Vice-President of Bioculture Puerto Rico, Inc., a corporation duly organized under the laws of the Commonwealth of Puerto Rico.
3. I have been authorized by Bioculture Puerto Rico, Inc. to enter into and execute any and all documents necessary for the application of the animal importation permit.
4. Bioculture Puerto Rico, Inc. is developing a primate center in the Municipality of Guayama with the purpose of breeding, weaning and growing of Specific Pathogen Free *Macaca Fascicularis* (also known as long tail macaque) to be prepared, supplied and exported for scientific purposes, mainly biomedical research.
5. The animals will only be prepared and supplied to Contract Research Organizations, pharmaceutical companies, federal or state government agencies and universities or educational institutions authorized to do biomedical research.

6. Article 8.01 of Regulation 6765 requires that the entity that requests an importation of animals permit be dedicated, totally or partially, to the importation of exotic animals for the purpose of scientific research, which is exclusively what Bioculture Puerto Rico, Inc. will do.

7. All of the above stated is the truth and nothing but the truth, as I know it to be from my personal knowledge.

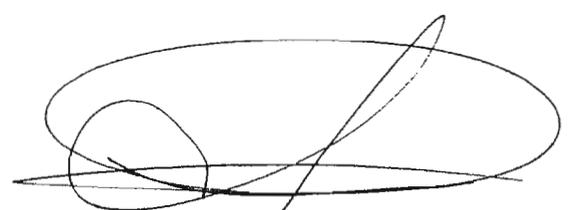
In San Juan, Puerto Rico on this 19 day of February, 2010.



MOSHE BUSHMITZ
VICE-PRESIDENTE
BIOCULTURE PUERTO RICO, INC.

Affidavit No. 4105

Sworn and signed before me by **DR. MOSHE BUSHMITZ**, of the above described personal circumstances, who is known to me personally, on this 19 day of February, 2010 in San Juan, Puerto Rico.



NOTARY PUBLIC





PRIDCO

COMMONWEALTH OF PUERTO RICO
Puerto Rico Industrial Development Company

May 13, 2010

Hon. Daniel Galán Kerkadó
Secretary
Natural Resources and Environment Department
PO Box 366147
San Juan, Puerto Rico 00936

MAY 13 2010

Re: Bioculture

Dear Secretary:

As discussed and agreed on our meeting that took place last Monday, May 10, 2010, we enclosed the supplementary information requested by the Natural Resources and Environment Department ("NRED") in order to issue the import license for scientific purposes under Section 8.06 of Regulation Number 6765. The enclosed information is the following:

1. A statement as to the effect that the purpose of importing the animals is solely scientific.
2. A brief description of the type of investigation that Bioculture's client will perform.
3. Amount, age and gender of the animals to be imported.
4. A statement of the benefits to the species.
5. Evidence of applications for permits with other regulatory agencies.
6. Evidence from the University of Puerto Rico, as well as other universities and research institutions establishing Bioculture's good standing in the scientific community.

COMMONWEALTH OF PUERTO RICO
Puerto Rico Industrial Development Company

As discussed in the meeting, the supplementary information will be part of the original application submitted by Bioculture.

Cordially,



Edgardo Arroyo, Esq.
Tax and Finance Consultant

Cc: Bushmitz Mark Moshe, DVM
Javier Vázquez Morales, Esq.

M.L. & R.E. LAW FIRM

513 JUAN J. JIMENEZ ST.
HATO REY, P.R. 00918
TEL (787) 999-2972 - FAX (787) 751-2221



16 de julio de 2010

Hon. Daniel J. Galán Kercadó
Secretario
Departamento de Recursos Naturales y Ambientales
P. O. Box 366147
San Juan, Puerto Rico 00936

Re: Enmienda al Permiso de Importación de Animales Núm. 2010-IC-O-VS-PVS15-SJ-00416-0502-2010.

Estimado Secretario Galán:

El pasado 25 de mayo, el Departamento de Recursos Naturales y Ambientales (en adelante "DRNA") expidió el permiso de referencia. Luego de revisar el mismo proponemos las siguientes enmiendas al permiso para su consideración. Las enmiendas aquí presentadas tienen el propósito de aclarar algunas condiciones y dar cumplimiento a las mismas balanceando la viabilidad de las operaciones de la empresa con el deber del DRNA de velar por el cumplimiento de la política pública del Estado en materia de conservación de vida silvestre.

Enmiendas al Permiso Número 2010-IC-O-VS-PVS15-SJ-00416-0502-2010¹

Previo a entrar en las condiciones del permiso señalamos que en el epígrafe del Permiso aparece el término "Expira" pero consigna la fecha de emisión. Suponemos que es un error clerical y que debe ser la fecha de emisión. Por lo tanto, se debe cambiar para que lea "Fecha de expedición: 26 de mayo de 2010". De otra parte, para que no haya dudas ni controversias sobre el tipo de permiso, entendiéndose que es un permiso de importación y exportación para fines científicos, en el epígrafe en la parte de "Tipo de Permiso" debe decir: "Importación y Exportación para Fines Científicos".

Ahora bien, atendiendo específicamente las condiciones del permiso, solicitamos las siguientes enmiendas:

Condición 1.5. - La redacción se presta a confusión ya que el límite de 4,500 individuos durante la vigencia del permiso es aplicable a la importación, no a la exportación. Para que esté claro, se propone la siguiente enmienda, que no altera el propósito de la condición:

¹ Se utilizara el mismo sistema de enumeración del permiso actual para facilitar la identificación de las condiciones a enmendarse.

Se autoriza a Bioculture PR, Inc. a importar y exportar para fines científicos, primates de la especie *Macaca fascicularis*. Entendiéndose que el número de animales a importar no excederá la capacidad autorizada por el Departamento de Agricultura de los Estados Unidos (USDA) de jaulas para mantener los animales; y la importación de animales para procrear no será más de cuatro mil quinientos (4,500) individuos durante el término de vigencia de este Permiso, según se desglosa a continuación:...

Condición 1.7.1. - Recomendamos la siguiente redacción de este inciso para propósitos de claridad sin que se afecte el resultado porque para todos los efectos, los autorizados por ley se refiere a entidades académicas de educación universitaria dedicadas a la investigación científicas en Puerto Rico:

Será ilegal vender, ceder o transferir los animales en cuestión a otra persona o entidad, dentro del territorio de Puerto Rico, excepto a las entidades autorizadas por ley dedicadas a investigación científica en Puerto Rico. De Bioculture PR, Inc. realizar cualquier venta, cesión o transferencia a cualquiera de éstas, deberá notificarlo al DRNA dentro de un término de treinta (30) días, a partir de la fecha en que se realice la transacción. La notificación deberá incluir el número de primates que hayan sido vendidos, cedidos o transferidos.

Condición 1.7.4. - De la misma no surge en que momento se debe entregar el certificado veterinario. Sugerimos que el certificado médico deberá conservarse en las facilidades de Bioculture y estar disponibles para producirse en casos de inspecciones o por solicitud oportuna del DRNA.

Condición 1.7.8 - La descripción de las medidas de seguridad se presta para confusión ya que el sistema de verjas, según el plano aprobado por ARPe incluye una verja electrificada con detectores de movimiento. Se debe mencionar también el sistema de triple seguro en las jaulas. Sugerimos el siguiente lenguaje:

La instalación contará con máxima seguridad. Lo anterior incluye, pero no se limita, a lo siguiente: verja alrededor del perímetro de las jaulas que deberá estar electrificada, jaulas con triple seguro para acceder a los animales, guardias de seguridad las veinticuatro (24) horas, alarmas contra escape y detectores de movimiento en la verja del perímetro conectado al sistema de alarmas.

Condiciones 1.7.10 y 1.7.11 - Bioculture está en disposición de adquirir el seguro de responsabilidad pública con el límite impuesto de un millón de dólares y los respectivos endosos sin la imposición de una fianza. Es evidente que la responsabilidad de los daños que causen los animales está cubierta por el seguro de responsabilidad pública,

por ello, no es necesaria la fianza en este renglón. El interés público no se vería afectado por este cambio pues habrá una aseguradora respondiendo, además de Bioculture. En resumidas cuentas, lo que se propone es que los límites escalonados descritos en el art. 1.7.12 sea el de la póliza de seguros, ya que ésta cubriría la responsabilidad pública. De lo contrario, sabemos que tal fianza no solo resultaría redundante, sino que, al no existir compañías dispuestas a vender tal producto, dicho requisito impediría que Bioculture pueda operar en Puerto Rico. está disponible y no haría viable la operación.

Condición 1.7.12. Modificar este artículo para atemperarlo a los cambios antes descritos sobre el seguro de responsabilidad pública y al establecimiento de un Fondo Especial según se sugiere a continuación:

Bioculture está dispuesto a establecer un Fondo Especial de ciento setenta mil dólares (\$170,000)² para asegurar aquellos gastos relacionados a la captura de animales en casos de fuga y manejo de los animales en casos de abandono o quiebra de Bioculture. Dicha suma de dinero se consignará en una cuenta PLICA y los fondos podrán ser retirados por el Gobierno de Puerto Rico, el DRNA o el Departamento de Agricultura según se describe a continuación:

1.7.12.1 - En aquellos casos relacionados a la fuga de animales (incluyendo casos de desastres naturales, causa mayor o negligencia atribuible a Bioculture), Bioculture deberá llevar a cabo gestiones para la captura inmediata del animal. Si en veinticuatro (24) horas no logra su captura, deberá notificarlo al DRNA. Aquellos gastos que incurra el DRNA para la captura de los animales deberá ser reembolsado a través de la compañía aseguradora y/o Bioculture. En caso de que la compañía aseguradora o Bioculture no cumplan con el reembolso de los gastos, el DRNA podrá desembolsar la cantidad correspondiente del Fondo Especial. Bioculture proveerá personal experto en captura de primates para asistir al DRNA.

1.7.12.2 - En caso de un abandono o quiebra de Bioculture donde no se dispusieran de los animales, se podrá utilizar el dinero en el Fondo Especial para que el DRNA lleve a cabo las gestiones necesarias para disponer de los mismos.

Condición 1.7.14 - Esta condición plantea un serio problema por implicar una renuncia a derechos constitucionales. Además, debe haber una razonabilidad en la intervención

² Esta cantidad esta basada en los más de 20 años de experiencia de Bioculture en el manejo y captura de animales. A pesar de que durante la existencia de la compañía no ha ocurrido fuga de animal alguno, Bioculture ha llevado operaciones de captura de los *Macaca Fascicularis* y conoce el trabajo que conlleva la captura de los mismos así como el equipo necesario para ejecutar dicha operación. [Ver Anejo 1] También se conocen los costos de disponer de los animales por razones de eutanasia. [Ver Anejo 2].

de forma que se eviten abusos o arbitrariedades. A tales efectos, el artículo debe leer:

El DRNA por conducto de la Unidad de Vida Silvestre del Cuerpo de Vigilantes, podrá en cualquier momento y sin previo aviso, entrar a las instalaciones de Bioculture PR, Inc., para inspeccionar que las condiciones de este Permiso se estén cumpliendo. Entendiéndose que dichas visitas para inspección no serán arbitrarias y caprichosas ni con frecuencia irrazonable. El empleado que realice la inspección, si desea acceder las áreas de los animales, deberá cumplir con las medidas de cuidado y seguridad requeridas por Bioculture y el Departamento de Agricultura de los Estados Unidos (USDA). Mediante la firma de este documento Bioculture PR, Inc. renuncia expresa pero limitadamente a cualquier reclamo de derecho contra registros irrazonables en cuanto a las inspecciones que puedan llevarse a cabo por el DRNA para verificar el cumplimiento con las condiciones de este Permiso pero con ningún otro registro.

Condición 1.7.16. - Los cambios que se sugieren es que se amplíe el tiempo que tiene Bioculture para capturar el animal evadido. Aunque con el diseño de los edificios de la operación y las medidas de seguridad que se habrán de tomar, no se prevé que hayan fugas, lo cierto es que cuatro (4) horas para capturar el animal constituye un término arbitrario e irrazonablemente corto. Además, no es necesario reclamar directamente al seguro si Bioculture tiene un plazo razonable para reembolsar los gastos. Por ello, recomendamos modificarlo para que lea:

En caso de que se escape cualquier primate por la razón que fuere, Bioculture PR, Inc. será responsable del rastreo y captura del mismo. Si dentro de veinticuatro (24) horas del escape, Bioculture PR, Inc. no ha podido atrapar el primate, tendrá la obligación de notificar este hecho al DRNA, quién se encargará de la captura del mismo y recobrará los costos de tal operación de conformidad con lo establecido en el art. 1.7.12, ante. De Bioculture PR, Inc. incumplir con la notificación, el Permiso podrá ser revocado.

Condición 1.7.18 - En este caso lo correcto sería el someter copia certificada de la póliza de seguro de responsabilidad pública. Debe, por tanto, leer:

Bioculture PR, Inc. será responsable de someter copia certificada de las pólizas de seguro de responsabilidad pública, por las cantidades establecidas anteriormente, con treinta días de anticipación a la fecha en que entre en vigor el segundo, tercero, cuarto y quinto año de vigencia del Permiso. El incumplimiento con esta disposición podrá dar lugar a la revocación de este Permiso.

Condición 1.7.20. - Se repite y/o contradice lo provisto en los artículos 1.7.1.7 y 1.7.13.
Para mayor claridad debe leer:

Este Permiso tendrá vigencia y operará en pleno vigor en la fecha de su expedición, condicionado a que en los próximos treinta (30) días de haber sido expedido, Bioculture PR, Inc. haga entrega al DRNA de copia certificada de las pólizas de seguro de responsabilidad pública y de la fianza de cumplimiento requerida para cada año de vigencia de este Permiso.

Con las enmiendas aquí contenidas Bioculture podrá llevar a cabo su operación satisfactoriamente garantizando el interés público de maximizar la seguridad del ambiente y los ciudadanos de Puerto Rico.

Estamos disponibles para discutir las enmiendas solicitadas a su conveniencia o aclarar cualquier duda sobre las mismas.

Sin otro particular quedo de usted.

Respetuosamente,



Emil Rodríguez Escudero



Bioculture (Puerto Rico) Inc.
Pueblita Carmen, Box HCO2 - 4250
Guayama
Puerto Rico 00734

Document BCM.PR.02.07

Animal Trapping Team

Bioculture has over 25 years experience in the trapping of wild (feral) monkeys on the island of Mauritius.

Trapping of monkeys requires a great knowledge of animal biology and behaviour.

The best trapping team should be comprised of an animal care taker who is very familiar with animal behaviour and an assistant to help place and maintain traps.

Equipment needed:

- Binoculars
- 4 individual traps that are baited with food and are triggered by the animal itself. These are placed near animal with its favorite food inside.
- Nets for animal trapping.
- Tranquilizing darts which can be fired or blown at the animal.
- Radio communication
- 4x4 vehicles.

A daily operation cost for a professional trapping team is about 200\$

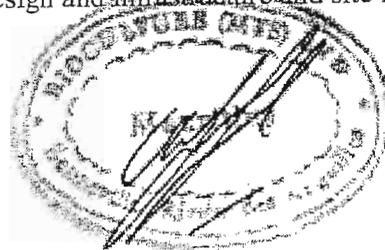
A monthly operation cost of a professional trapping team is about 5000\$

A trapping team will cover a very large area per day searching for animals.

It should however be remembered that:

1. Animals as a general rule will not venture far from the farm site in the event of an escape, and are thus easily recaptured.
2. Bioculture has invested very significantly in cage design and infrastructure and site fencing to prevent escapes.

Owen Griffiths
Managing Director.





BIOCULTURE
LABORATORIOS

Bioculture (Puerto Rico) Inc.
 Pucblita Carmen, Bos HCO2 - 4250
 Guayama
 Puerto Rico 00784

Document BCM.PR.01.07

Euthanasia of animals

- ⇒ Animals are euthanized by an injection of concentrated anesthetic.
- ⇒ The Material is given based on the body weight and the quantity for adult animal is 4-5 kg of body weight (like a cat).
- ⇒ The price of a dose to anesthetic to euthanize one animal is between \$1 and \$2.
- ⇒ Procedure to be carried out by veterinarian with animal technician.
- ⇒ A veterinarian can euthanize a very large number of animals per day if needed.
- ⇒ In animal shelters veterinarians can euthanize hundreds of animals per day.
- ⇒ We estimate the cost for one animal with its disposal to be not more then \$50 and the price will drop sharply if the quantity is much bigger.

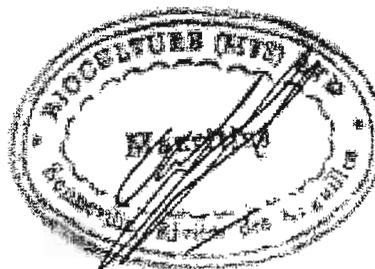
Cost table for an euthanasia procedure.

Time/manpower for procedure: 1 animal technician and 1 veterinarian for 2 mins (time taken for procedure)

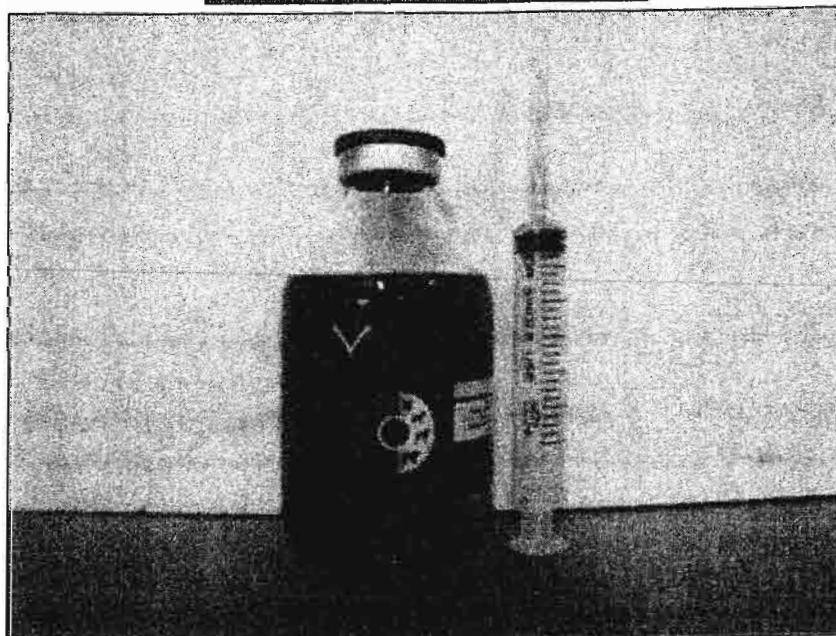
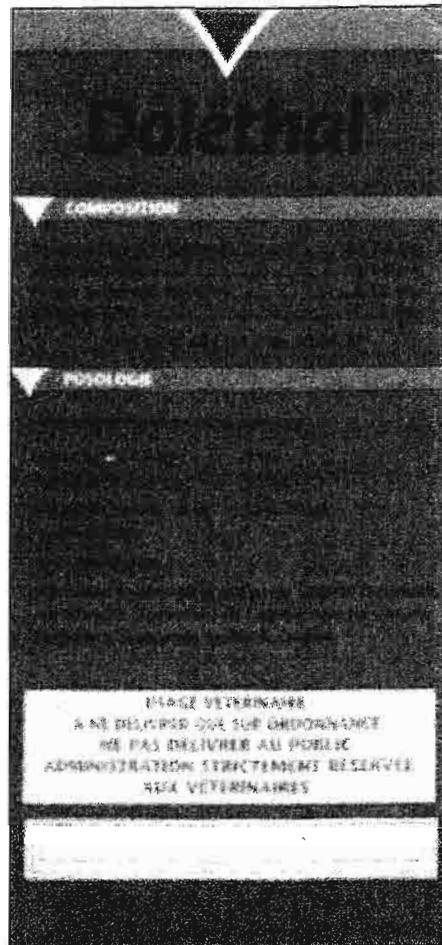
PROCEDURE: EUTHANASIA

	AMOUNT	COST (\$)	
TIME	2 mins		
MANPOWER			
Handler/Technician	1		
Veterinarian	1		
ITEMS			
Syringe+ Needle	2	0.50	
Latex Gloves	8	0.30	
Ketamine	0.4 ml	0.30	
Dolethal	4 ml	1.00	
Disposal Bag	1	0.50	
TOTAL		\$2.60	

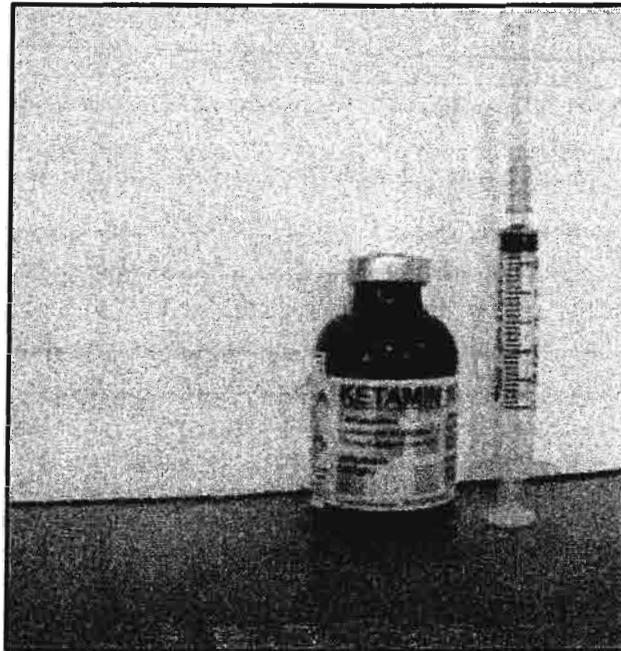
All calculations based on a 4 kg animal
 Disposable items plus medicine costs based on \$US costs in Mauritius.



Euthanasia drugs



Tranquilizers



KETAMIN 10% INJ

Contraindications:

Contraindicated in patients with severe hypertension, hyperthyroidism, and severe heart disease.

Indications:

Used for the induction and maintenance of general anesthesia in patients with normal airway reflexes and cardiovascular stability. It is used for the induction and maintenance of general anesthesia in patients with normal airway reflexes and cardiovascular stability. It is used for the induction and maintenance of general anesthesia in patients with normal airway reflexes and cardiovascular stability.

Contraindications:

Contraindicated in patients with severe hypertension, hyperthyroidism, and severe heart disease.

Dosage and administration:

For intramuscular injection:

Adults: 1-2 mg/kg (0.1-0.2 mg/kg) for induction of anesthesia; 0.5-1 mg/kg for maintenance.

Children: 1-2 mg/kg (0.1-0.2 mg/kg) for induction of anesthesia; 0.5-1 mg/kg for maintenance.

Infants: 1-2 mg/kg (0.1-0.2 mg/kg) for induction of anesthesia; 0.5-1 mg/kg for maintenance.

Neonates: 1-2 mg/kg (0.1-0.2 mg/kg) for induction of anesthesia; 0.5-1 mg/kg for maintenance.

Geriatric: 1-2 mg/kg (0.1-0.2 mg/kg) for induction of anesthesia; 0.5-1 mg/kg for maintenance.

Renal impairment: 1-2 mg/kg (0.1-0.2 mg/kg) for induction of anesthesia; 0.5-1 mg/kg for maintenance.

Hepatic impairment: 1-2 mg/kg (0.1-0.2 mg/kg) for induction of anesthesia; 0.5-1 mg/kg for maintenance.

Side effects:

Common side effects include: increased heart rate, increased blood pressure, increased respiratory rate, and increased oxygen consumption. Other side effects include: nausea, vomiting, and hallucinations. The drug is also known to cause a dissociative state, which is characterized by a feeling of detachment from the body and surroundings.

Precautions:

Use with caution in patients with severe hypertension, hyperthyroidism, and severe heart disease. Avoid use in patients with severe respiratory depression.

Interactions:

Use with caution in patients with severe hypertension, hyperthyroidism, and severe heart disease. Avoid use in patients with severe respiratory depression.

Administration:

Inject intramuscularly into the gluteal muscle. Avoid use in patients with severe respiratory depression.

Storage conditions:

Store in a cool, dry place. Avoid exposure to light and moisture. Do not use if the solution is cloudy or contains a precipitate.

Warnings:

Use with caution in patients with severe hypertension, hyperthyroidism, and severe heart disease. Avoid use in patients with severe respiratory depression.

M.L. & R.E. LAW FIRM

513 JUAN J. JIMENEZ ST.
HATO REY, P.R. 00918
TEL (787) 999-2972 - FAX (787) 751-2221

15 de julio de 2010

Hon. Daniel J. Galán Kercadó
Secretario
Departamento de Recursos Naturales y Ambientales
P. O. Box 366147
San Juan, Puerto Rico 00936

Re: Enmienda al Permiso de Importación de Animales Núm. 2010-IC-O-VS-PVS15-SJ-00416-0502-2010.

Estimado Secretario Galán:

El pasado 25 de mayo, el Departamento de Recursos Naturales y Ambientales (en adelante "DRNA") expidió el permiso de referencia. Luego de revisar el mismo proponemos las siguientes enmiendas al permiso para su consideración. Las enmiendas aquí presentadas tienen el propósito de aclarar algunas condiciones y dar cumplimiento a las mismas balanceando la viabilidad de las operaciones de la empresa con el deber del DRNA de velar por el cumplimiento de la política pública del Estado en materia de conservación de vida silvestre.

Enmiendas al Permiso Número 2010-IC-O-VS-PVS15-SJ-00416-0502-2010¹

Previo a entrar en las condiciones del permiso señalamos que en el epígrafe del Permiso aparece el término "Expira" pero consigna la fecha de emisión. Suponemos que es un error clerical y que debe ser la fecha de emisión. Por lo tanto, se debe cambiar para que lea "Fecha de expedición: 26 de mayo de 2010". De otra parte, para que no haya dudas ni controversias sobre el tipo de permiso, entendiéndose que es un permiso de importación y exportación para fines científicos, en el epígrafe en la parte de "Tipo de Permiso" debe decir: "Importación y Exportación para Fines Científicos".

Ahora bien, atendiendo específicamente el las condiciones del permiso, solicitamos las siguientes enmiendas:

Condición 1.5. - La redacción se presta a confusión ya que e límite de 4,500 individuos durante la vigencia del permiso es aplicable a la importación, no a la exportación. Para que esté claro, se propone la siguiente enmienda, que no altera el propósito de la condición:

¹ Se utilizara el mismo sistema de enumeración del permiso actual para facilitar la identificación de las condiciones a enmendarse.

Se autoriza a Bioculture PR, Inc. y personal autorizado, a importar y exportar para fines científicos, primates de la especie *Macaca fascicularis*. Entendiéndose que la importación será hasta un máximo de cuatro mil quinientos (4,500) individuos, durante el término de vigencia de este Permiso, según se desglosa a continuación: ...

Condición 1.7.1. - Recomendamos la siguiente redacción de este inciso para propósitos de claridad sin que se afecte el resultado porque para todos los efectos, los autorizados por ley se refiere a entidades académicas de educación universitaria dedicadas a la investigación científicas en Puerto Rico:

Será ilegal vender, ceder o transferir los animales en cuestión a otra persona o entidad, dentro del territorio de Puerto Rico, excepto a las entidades autorizadas por ley dedicadas a investigación científica en Puerto Rico. De Bioculture PR, Inc. realizar cualquier venta, cesión o transferencia a cualquiera de éstas, deberá notificarlo al DRNA dentro de un término de treinta (30) días, a partir de la fecha en que se realice la transacción. La notificación deberá incluir el número de primates que hayan sido vendidos, cedidos o transferidos.

Condición 1.7.4. - De la misma no surge en que momento se debe entregar el certificado veterinario. Sugerimos que el certificado médico deberá producirse mediante solicitud a tales efectos por el DRNA.

Condición 1.7.8 - La descripción de las medidas de seguridad se presta para confusión ya que el sistema de verjas, según el plano aprobado por ARPe incluye una verja electrificada con detectores de movimiento. Se debe mencionar también el sistema de triple seguro en las jaulas. Sugerimos el siguiente lenguaje:

La instalación contará con máxima seguridad. Lo anterior incluye, pero no se limita, a lo siguiente: verja alrededor del perímetro de las jaulas que deberá estar electrificada, jaulas con triple seguro para acceder a los animales, guardias de seguridad las veinticuatro (24) horas, alarmas contra escape y detectores de movimiento en la verja del perímetro conectado al sistema de alarmas.

Condiciones 1.7.10 y 1.7.11 - Bioculture está en disposición de adquirir el seguro de responsabilidad pública con el límite impuesto de un millón de dólares y los respectivos endosos sin la imposición de una fianza. Es evidente que la responsabilidad de los daños que causen los animales está cubierta por el seguro de responsabilidad pública, por ello, no es necesaria la fianza en este renglón. El interés público no se vería afectado por este cambio pues habrá una aseguradora respondiendo, además de Bioculture. En resumidas cuentas, lo que se propone es que los límites escalonados descritos en el art. 1.7.12 sea el de la póliza de seguros, ya que ésta cubriría la

responsabilidad pública. De lo contrario, sabemos que tal fianza no solo resultaría redundante, sino que, al no existir compañías dispuestas a vender tal producto, dicho requisito impediría que Bioculture pueda operar en Puerto Rico. está disponible y no haría viable la operación.

Condición 1.7.12. Modificar este artículo para atemperarlo a los cambios antes descritos sobre el seguro de responsabilidad pública y al establecimiento de un Fondo Especial según se sugiere a continuación:

Bioculture está dispuesto a establecer un Fondo Especial de ciento setenta mil dólares (\$170,000)² para asegurar aquellos gastos relacionados a la captura de animales en casos de fuga y manejo de los animales en casos de abandono o quiebra de Bioculture. Dicha suma de dinero se consignará en una cuenta PLICA y los fondos podrán ser retirados por el Gobierno de Puerto Rico, el DRNA o el Departamento de Agricultura según se describe a continuación:

1.7.12.1 - En aquellos casos relacionados a la fuga de animales (incluyendo casos de desastres naturales, causa mayor o negligencia atribuible a Bioculture), Bioculture deberá llevar a cabo gestiones para la captura inmediata del animal. Si en veinticuatro (24) horas no logra su captura, deberá notificarlo al DRNA. Aquellos gastos que incurra el DRNA para la captura de los animales deberá ser reembolsado a través de la compañía aseguradora y/o Bioculture. En caso de que la compañía aseguradora o Bioculture no cumplan con el reembolso de los gastos, el DRNA podrá desembolsar la cantidad correspondiente del Fondo Especial. Bioculture proveerá personal experto en captura de primates para asistir al DRNA.

1.7.12.2 - En caso de un abandono o quiebra de Bioculture donde no se dispusieran de los animales, se podrá utilizar el dinero en el Fondo Especial para que el DRNA lleve a cabo las gestiones necesarias para disponer de los mismos.

Condición 1.7.14 - Esta condición plantea un serio problema por implicar una renuncia a derechos constitucionales. Además, debe haber una razonabilidad en la intervención de forma que se eviten abusos o arbitrariedades. A tales efectos, el artículo debe leer:

El DRNA por conducto de la Unidad de Vida Silvestre del Cuerpo de Vigilantes, podrá en cualquier momento y sin previo

² Esta cantidad esta basada en los más de 20 años de experiencia de Bioculture en el manejo y captura de animales. A pesar de que durante la existencia de la compañía no ha ocurrido fuga de animal alguno, Bioculture ha llevado operaciones de captura de los *Macaca Fascicularis* y conoce el trabajo que conlleva la captura de los mismos así como el equipo necesario para ejecutar dicha operación. [Ver Anejo 1] También se conocen los costos de disponer de los animales por razones de eutanasia. [Ver Anejo 2].

aviso, entrar a las instalaciones de Bioculture PR, Inc., para inspeccionar que las condiciones de este Permiso se estén cumpliendo. Entendiéndose que dichas visitas para inspección no serán arbitrarias y caprichosas ni con frecuencia irrazonable. El empleado que realice la inspección, si desea acceder las areas de los animales, deberá cumplir con las medidas de cuidado y seguridad requeridas por Bioculture y el Departamento de Agricultura de los Estados Unidos (USDA). Mediante la firma de este documento Bioculture PR, Inc. renuncia expresa pero limitadamente a cualquier reclamo de derecho contra registros irrazonables en cuanto a las inspecciones que puedan llevarse a cabo por el DRNA para verificar el cumplimiento con las condiciones de este Permiso pero con ningún otro registro.

Condición 1.7.16. - Los cambios que se sugieren es que se amplíe el tiempo que tiene Bioculture para capturar el animal evadido. Aunque con el diseño de los edificios de la operación y las medidas de seguridad que se habrán de tomar, no se prevé que hayan fugas, lo cierto es que cuatro (4) horas para capturar el animal constituye un término arbitrario e irrazonablemente corto. Además, no es necesario reclamar directamente al seguro si Bioculture tiene un plazo razonable para reembolsar los gastos. Por ello, recomendamos modificarlo para que lea:

En caso de que se escape cualquier primate por la razón que fuere, Bioculture PR, Inc. será responsable del rastreo y captura del mismo. Si dentro de veinticuatro (24) horas del escape, Bioculture PR, Inc. no ha podido atrapar el primate, tendrá la obligación de notificar este hecho al DRNA, quién se encargará de la captura del mismo y recobrará los costos de tal operación de conformidad con lo establecido en el art. 1.7.12, ante. De Bioculture PR, Inc. incumplir con la notificación, el Permiso podrá ser revocado.

Condición 1.7.18 - En este caso lo correcto sería el someter copia certificada de la póliza de seguro de responsabilidad pública. Debe, por tanto, leer:

Bioculture PR, Inc. será responsable de someter copia certificada de las pólizas de seguro de responsabilidad pública, por las cantidades establecidas anteriormente, con treinta días de anticipación a la fecha en que entre en vigor el segundo, tercero, cuarto y quinto año de vigencia del Permiso. El incumplimiento con esta disposición podrá dar lugar a la revocación de este Permiso.

Condición 1.7.20. - Se repite y/o contradice lo provisto en los artículos 1.7.1.7 y 1.7.13. Para mayor claridad debe leer:

Este Permiso tendrá vigencia y operará en pleno vigor en la fecha de su expedición, condicionado a que en los próximos treinta (30) días de haber sido expedido, Bioculture PR, Inc. haga entrega al DRNA de copia certificada de las pólizas de seguro de responsabilidad pública y de la fianza de cumplimiento requerida para cada año de vigencia de este Permiso.

Con las enmiendas aquí contenidas Bioculture podrá llevar a cabo su operación satisfactoriamente garantizando el interés público de maximizar la seguridad del ambiente y los ciudadanos de Puerto Rico.

Estamos disponibles para discutir las enmiendas solicitadas a su conveniencia o aclarar cualquier duda sobre las mismas.

Sin otro particular quedo de usted.

Respetuosamente,



Emil Rodríguez Escudero



Bioculture (Puerto Rico) Inc.
 Puchlita Carmen Box H002 - 4150
 GUAYAMA
 Puerto Rico 00734

Document BCM.PR.02.07

Animal Trapping Team

Bioculture has over 25 years experience in the trapping of wild (feral) monkeys on the island of Mauritius.

Trapping of monkeys requires a great knowledge of animal biology and behaviour.

The best trapping team should be comprised of an animal care taker who is very familiar with animal behaviour and an assistant to help place and maintain traps.

Equipment needed:

- Binoculars
- 4 individual traps that are baited with food and are triggered by the animal itself. These are placed near animal with its favorite food inside.
- Nets for animal trapping.
- Tranquilizing darts which can be fired or blown at the animal.
- Radio communication
- 4x4 vehicles.

A daily operation cost for a professional trapping team is about 200\$

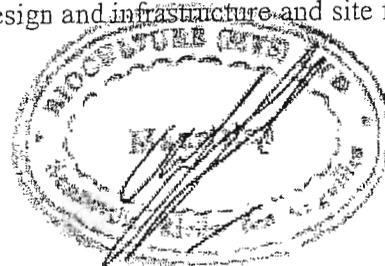
A monthly operation cost of a professional trapping team is about 5000\$

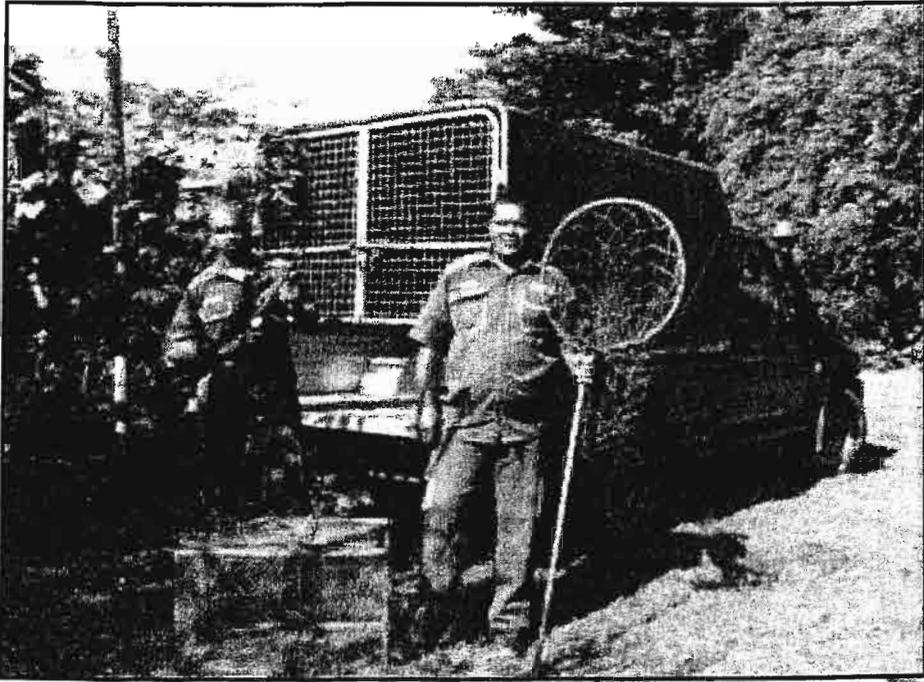
A trapping team will cover a very large area per day searching for animals.

It should however be remembered that:

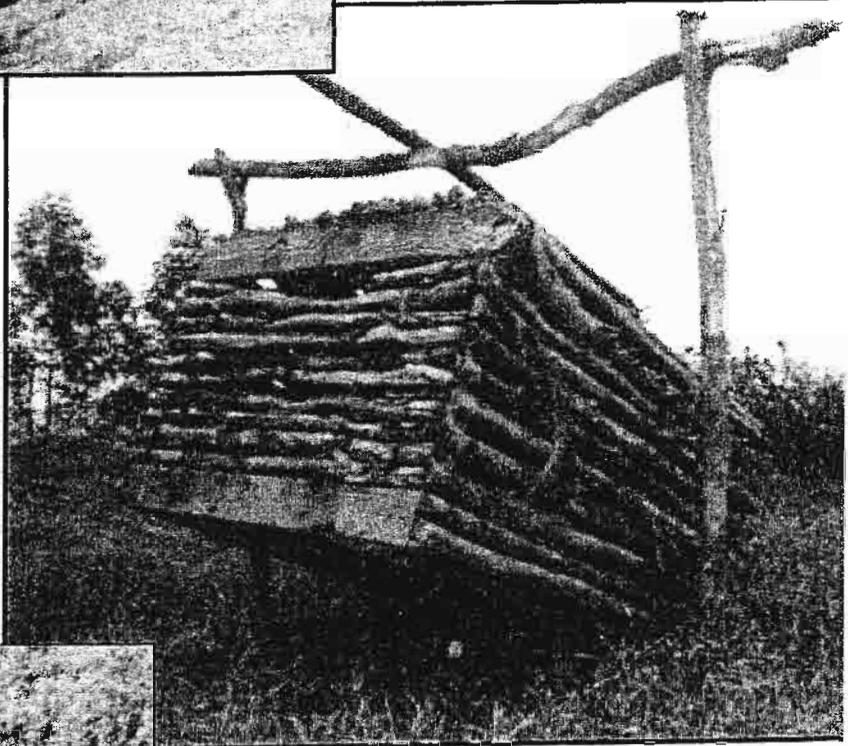
1. Animals as a general rule will not venture far from the farm site in the event of an escape, and are thus easily recaptured.
2. Bioculture has invested very significantly in cage design and infrastructure and site fencing to prevent escapes.

Owen Griffiths
 Managing Director.





Trappers, Net, dart gun and transport cage





Bioculture (Puerto Rico) Inc.
 Fuchina Carmen, Box HCO2 - 4250
 Guayama
 Puerto Rico 00784

Document BCM.PR.01.07

Euthanasia of animals

- ⇒ Animals are euthanized by an injection of concentrated anesthetic.
- ⇒ The Material is given based on the body weight and the quantity for adult animal is 4-5 kg of body weight (like a cat).
- ⇒ The price of a dose to anesthetic to euthanize one animal is between \$1 and \$2.
- ⇒ Procedure to be carried out by veterinarian with animal technician.
- ⇒ A veterinarian can euthanize a very large number of animals per day if needed.
- ⇒ In animal shelters veterinarians can euthanize hundreds of animals per day.
- ⇒ We estimate the cost for one animal with its disposal to be not more then \$50 and the price will drop sharply if the quantity is much bigger.

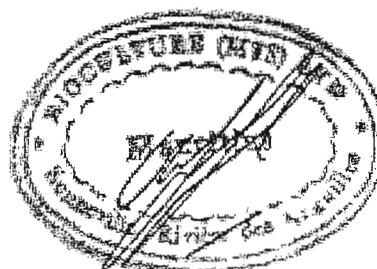
Cost table for an euthanasia procedure.

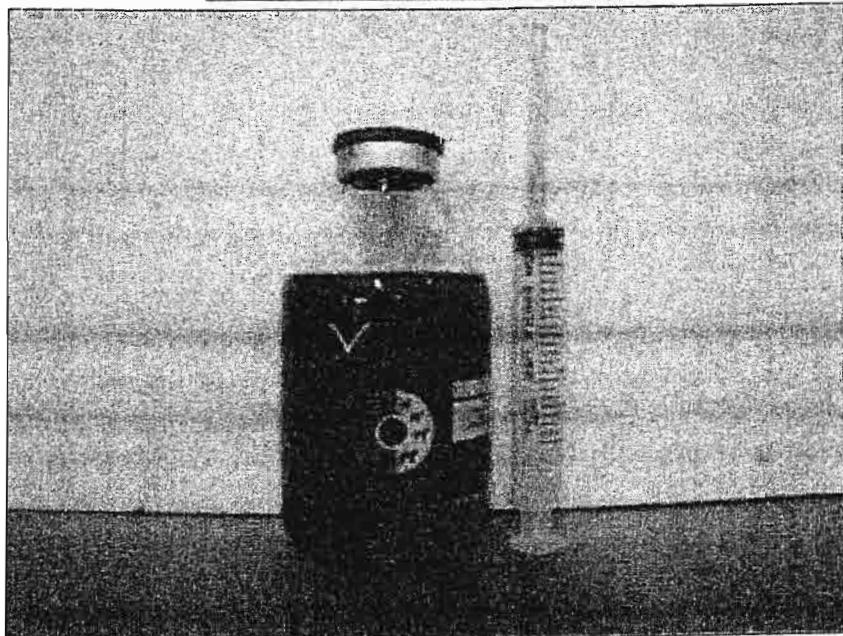
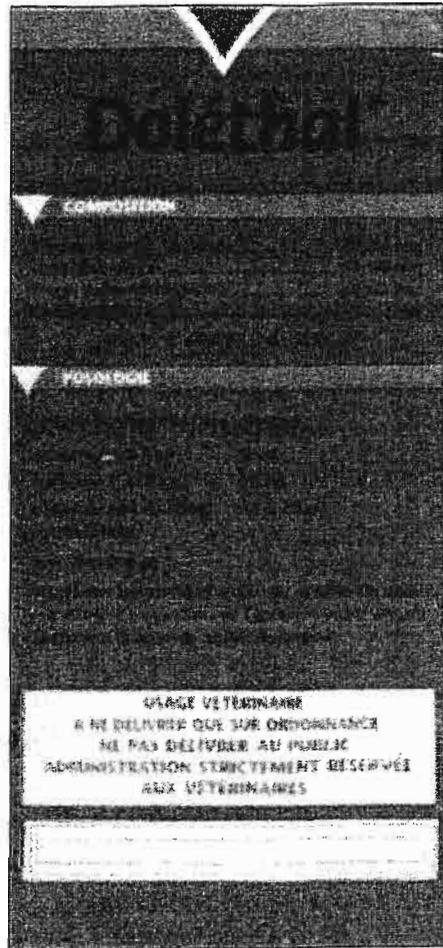
Time/manpower for procedure: 1 animal technician and 1 veterinarian for 2 mins (time taken for procedure)

PROCEDURE: EUTHANASIA

	AMOUNT	COST (\$)	
TIME	2 mins		
MANPOWER			
Handler/Technician	1		
Veterinarian	1		
ITEMS			
Syringe+ Needle	2	0.50	
Latex Gloves	8	0.30	
Ketamine	0.4 ml	0.30	
Dolethal	4 ml	1.00	
Disposal Bag	1	0.50	
TOTAL		\$2.60	

All calculations based on a 4 kg animal
 Disposable items plus medicine costs based on \$US costs in Mauritius.





Joshua M. Galarza

From: Emil Rodriguez [emil@mlrelaw.com]
Sent: Monday, August 09, 2010 4:30 PM
To: Joshua M. Galarza
Subject: Enmiendas Permiso Importación

Estimado Lcdo. Galarza,

17.11 A continuación le incluyo el lenguaje sugerido para la sección del Fondo Especial a nombre del DRNA.

Bioculture establecerá un Fondo Especial de cuatrocientos mil dólares (\$400,000) en una cuenta de plica para sufragar aquellos gastos relacionados a la captura de animales en casos de fuga y/o al manejo de los primates en la eventualidad que Bioculture abandone las facilidades o quiebre sin haber dispuesto de los primates. El Fondo Especial será creado mediante depósitos escalonados en la cuenta plica de la siguiente manera:

a) Un depósito inicial de doscientos mil dólares (\$200,000), el que deberá llevarse a cabo en o antes de los 30 días anteriores a la importación de los primeros primates para procrear. Esta suma será suficiente para los primeros dos años de vigencia del permiso.

b) Treinta (30) días antes del comienzo del tercer año del permiso, Bioculture deberá haber depositado la cantidad adicional de cien mil dólares (\$100,000) al Fondo Especial para un mantener un total de trescientos mil dólares (\$300,000).

c) Treinta (30) días antes del comienzo del cuarto año del permiso, Bioculture deberá haber depositado la cantidad adicional de cien mil dólares (\$100,000) al Fondo Especial para un total de cuatrocientos mil dólares (\$400,000).

El dinero depositado en la cuenta plica podrá ser utilizado por el Gobierno de Puerto Rico, el DRNA o el Departamento de Agricultura cuando ocurra alguno de los siguientes eventos:

1.7.12.1 - Cuando ocurra la fuga de primates durante la operación de Bioculture, (incluyendo casos de desastres naturales, fuerza mayor o negligencia atribuible a Bioculture), Bioculture deberá llevar a cabo gestiones para la captura inmediata del animal. Si en las próximas

veinticuatro (24) horas a partir de la fuga no logra su captura, deberá notificarlo al DRNA. Luego de esas veinticuatro (24) horas, aquellos gastos razonables y propios en los que incurra el DRNA para la captura del primate deberán ser reembolsados al DRNA, por la compañía aseguradora de Bioculture o por ésta misma, conforme a la factura que a tales efectos someta el DRNA a Bioculture . En caso de que la compañía aseguradora o Bioculture no cumplan con el reembolso de los gastos, el DRNA podrá retirar la cantidad facturada del Fondo Especial. Bioculture proveerá personal experto en captura de primates para asistir al DRNA.

1.7.12.2 - En caso de que Bioculture abandone las facilidades o presente una petición de liquidación ante la Corte de Quiebras y como consecuencia de ello abandone los primates y/o no disponga de ellos, las gestiones llevadas a cabo por el DRNA para la disposición de los primates serán pagadas por Bioculture y/o su aseguradora. En la eventualidad de que no ocurra ese pago, el DRNA podrá retirar la suma equivalente a los gastos razonables y necesarios apra disponer de los primates de la cuenta plica antes mencionada.

Le recuerdo también que, según solicitado por Bioculture, las cantidades de primates para procrear a importarse por año (Condiciones 1.5.1 a 1.5.5 del permiso actual) son las siguientes:

- 1.5.1 Mil (1,000) animales el primer año
- 1.5.2 Mil (1,000) animales el segundo año
- 1.5.3 Mil (1,000) animales el tercer año
- 1.5.4 Mil (1,000) animales el cuarto año
- 1.5.5 Quinientos (500) animales el quinto año

Agradeceré que se comunique conmigo para discutir los presentes cambios.

Cordialmente,

Emil Rodríguez Escudero
ML & RE Law Firm
513 Juan J. Jiménez St.
San Juan, P.R. 00918
emil@mlrelaw.com
Tel. (787) 999-2972
Fax (787) 751-2221

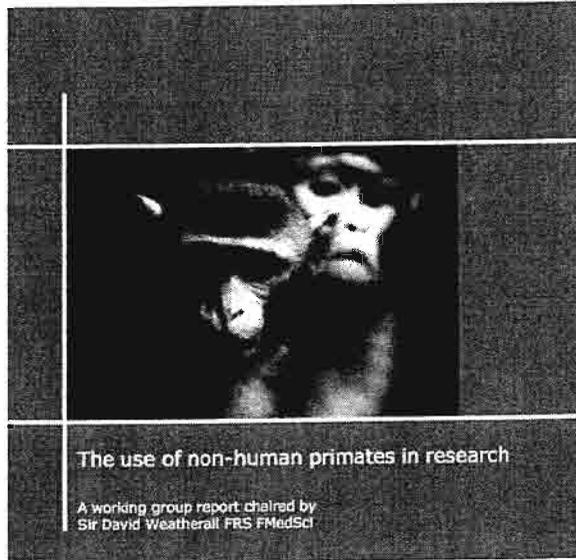
Bioculture Puerto Rico Inc.



Presentation to DNER

- ***Primates Are essential for Biomedical research and for drug development industry.***
- ***Most vaccines are developed and tested on primates.***
- ***Research of diseases like HIV , Parkinson's , diabetes, MS , West Nile, Dengue fever - is done with primates.***

UK government committee after almost 2 years declared that :
“primates are essential for biomedical research.”



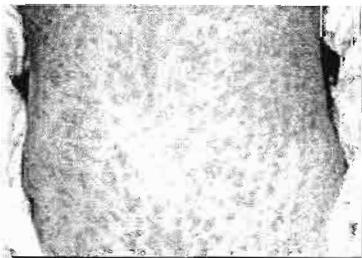
Smallpox eradication



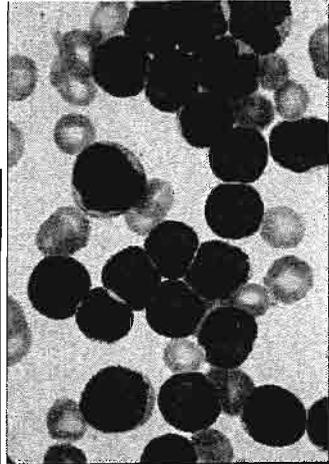
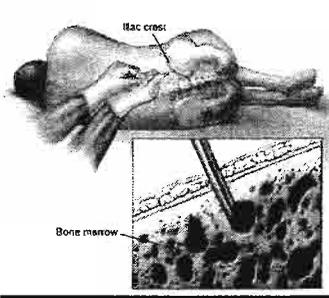
Polio eradication



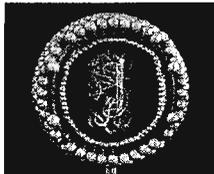
Measles , Rubella, Pertussis



Leukemia



HIV



Parkinson



Primate facility worldwide

- *Primate breeding farms exist in many countries in Europe , in the US , Japan , Canada and even in Puerto Rico.*
- *More then 220,000 are kept in more then 100 primates centers and labs.*

NIH
National primate
center
Wisconsin

WISCONSIN
NATIONAL PRIMATE RESEARCH CENTER
UNIVERSITY OF WISCONSIN-MADISON



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Intrame

The Wisconsin National Primate Research Center is one of eight federally supported (NIH-NCRB) National Primate Research Centers and the only one in the Midwest. More than 250 center scientists, through competitive grants, conduct research in primate biology with relevance to human and animal health.

The Primate Center is based in the Graduate School of the University of Wisconsin-Madison. The Center has strong research and teaching links to the UW Schools or Colleges of Medicine, Letters and Science, Agriculture and Life Sciences, and Veterinary Medicine. The Center is AAALAC accredited and its policies adhere to the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.

- Primate Center mission and objectives
- Primate Center discoveries
- How to find the Primate Center
- UW student admissions and the Primate Center

PRIMATE CENTER NEWS:

Nov. 20, 2007: Peeking in on the workings of an anxious brain

Nov. 20, 2007: UW-Madison scientists study human skin cells to embryonic state

Nov. 5, 2007: Horvitz wins Jacobson conservation award

October 8, 2007: Primate study shows excess vitamin A can be stored during fetal development

June 13, 2007: 2008-2009 WNPAC Venture Research Projects Invited

April 13, 2007: Walking Toward a Cure for Parkinson's Disease

NIH
National primate
center
Tulane



TULANE NATIONAL PRIMATE RESEARCH CENTER

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Microscopic

From the work of researchers at Tulane

The Tulane National Primate Research Center has a national mission to improve human and animal health through basic and applied biomedical research

To accomplish this mission the TNPRC: Conducts basic and applied biomedical research on human health problems using nonhuman primate models Investigates nonhuman primate biology and diseases with particular regard to the study of human health problems. Serves as a regional and national resource and center of excellence for biomedical research using nonhuman primates. Provides training for graduate students, postdoctoral fellows, veterinarians, undergraduates, veterinary students and visiting scientists. Educates the general public about the critical link between basic research with animal models and improvements in human health.

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Animal Research Helps People and Animals. Virtually everyone alive today has benefited from the medical advances made possible through animal research. Find out more...

Tulane University

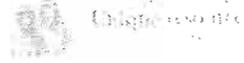
The Tulane National Primate Research Center is a division of Tulane University. Disclaimer

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NIH
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Yerkes



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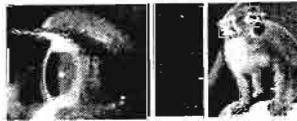
Science Starts Here

Highlighting Search Terms: national primate center [Search Again](#)

Why do some people develop neurodegenerative diseases while others do not? Do hormones play a role in social behavior? How can new treatments slow or stop the progression of infectious and noninfectious diseases?

Science begins with questions, and researchers at the Yerkes National Primate Research Center are taking the first steps in the comprehensive yet complicated process of finding answers. Our works in understanding the human body and behavior and in beginning the translational research process. Research started at Yerkes provides a vital connection to further scientific discovery that will improve the health of our nation and the world.

Seeing the Brain in a New Light



The brain is one of the last great frontiers of biology. This is because so much remains to be learned about how it functions, and interacts with the entire body and also because of new technology so, such as brain imaging, that the Yerkes primate scientists, a window into the active brain

Recent News

11/15/07
[Yerkes Researchers Recognize State of Emergency in Nonhuman Primates](#)

11/06/07
[Yerkes Researchers Present at 57th Annual Society for Neuroscience Conference](#)

10/29/07
[Oxysterol Fertilizes Dopamine Receptors Similar in Chimpanzees and Humans](#)

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Recent Coverage

[Michael J. Fox Foundation Awards \\$4.4 Million for Development of New Class of Parkinson's Therapy](#)

[Dr. Yolande Smith a Member of Multidisciplinary Team of](#)

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Southwest National Primate Research Center

Our Mission: To improve the health of our global community through innovative biomedical research with nonhuman primates.

In 1999, the Southwest National Primate Research Center (SNPRC) became the first new NCRR-funded National Primate Research Center (NPRC) in over 35 years. The SNPRC brings a number of unique strengths to the NPRC program, stemming from a long, productive history of nonhuman primate research at its host institution, the Southwest Foundation for Biomedical Research (SFBR). These unique strengths include the world's largest captive baboon population, the world's largest and best-characterized pedigreed primate population, the world's largest group of geneticists committed to research with and management of captive nonhuman primates, one of the largest nonhuman primate censuses of any NPRC, the largest chimpanzee census of any NPRC, the capacity for nonhuman primate studies in Bioscontainment Level 4, and a veterinary technical staff experienced in the management and use of nonhuman primates ranging from chimpanzees to marmosets.

John L. Vandenberg, Scientist and Director, SNPRC
Southwest National Primate Research Center

- Doctoral Staff**
- Jonathan S. Allen
 - Anthony Comuzzie
 - Laura A. Cox
 - Thomas Fagels
 - Leslie D. Goedert
 - Robert E. Lanford
 - Michael C. Mahoney
 - Jane Pecotte

We provide broad services in primate research to the southwestern region of the country, and serve the entire country with specialized technologies, capabilities, and primate resources, many of which are unique to the SNPRC. We provide services and conduct technical procedures requested by outside investigators participating in collaborative projects.

Baboons, SPF Indian-origin rhesus macaques, and marmosets are often available from our breeding colonies to sell to outside investigators who want to conduct research with them at the SNPRC. Most of the rhesus macaques are produced under support of an NIH grant aimed at providing these animals for AIDS-related research, although other research uses of this species also are possible. Baboons are generally available for sale and removal to other research facilities, and rhesus macaques and marmosets are occasionally available for sale. Click here to submit a request.

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National primate
center
Washington

Washington National Primate Research Center

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3rd International Conference on Primates Cognition

MISSION: TO PROVIDE THE APPROPRIATE ENVIRONMENT
TO SUPPORT OUTSTANDING BIOMEDICAL RESEARCH
ORIENTED TOWARDS SIGNIFICANT HUMAN HEALTH ISSUES
AND NONHUMAN PRIMATE HEALTH AND BIOLOGY

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Puerto
Rico



Funded by a grant from the National Center for Research Resources (NCRR)
(5P40RR03640),
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Staff

Cayo Santiago

Sabana Seca

Virology
Laboratory

Laboratory for
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(LPMG)

Recent
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CPRC
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(1993-2005)

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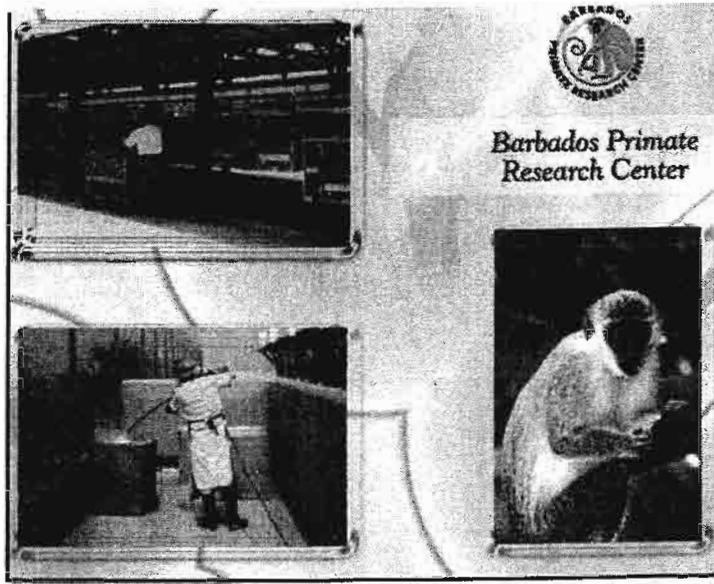
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Caribbean Primate Research Center

The Caribbean Primate Research Center (CPRC) is a research, training and education unit of the University of Puerto Rico (UPR), Medical Sciences Campus (MSC). The CPRC is supported by a core grant from the National Institutes of Health (NIH), National Center for Research Resources (NCRR), and the UPR. The CPRC has vast experience in the establishment and maintenance of rhesus macaque (*Macaca mulatta*) breeding colonies. The greatest strength of the CPRC is in conducting multidisciplinary, collaborative studies on the entire life cycle of rhesus monkeys as a biological model for humans. The animal care program of the CPRC is in full compliance with the directives established by the Animal Welfare Act (AWA). The program is monitored by the Institutional Animal Care and Use Committee (IACUC) of the UPR-MSC and the Animal Plant Health Inspection Services (APHIS) of the USDA. Since 1992, the CPRC has been fully accredited by the American Association for Accreditation of Laboratory Animal Care International (AAALAC).

Primate
research
center
Barbados



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Live Animal Division

The Live Animal Division (LAD) offers a wide range of services in addition to supply sources for live animals:

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- Pre-delivery Screening
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Better primates. Better products.

Welcome to Primate Products, Inc.

Primate Products, Inc. welcomes you to the opportunity to interact with the world's leading developer and distributor of enrichment devices, handling equipment, quality housing systems, and many other services designed specifically for nonhuman primates.

We encourage you to take the time to thoroughly explore our web site. You will see that we have a lot to offer to assist you in improving the lives of nonhuman primates.

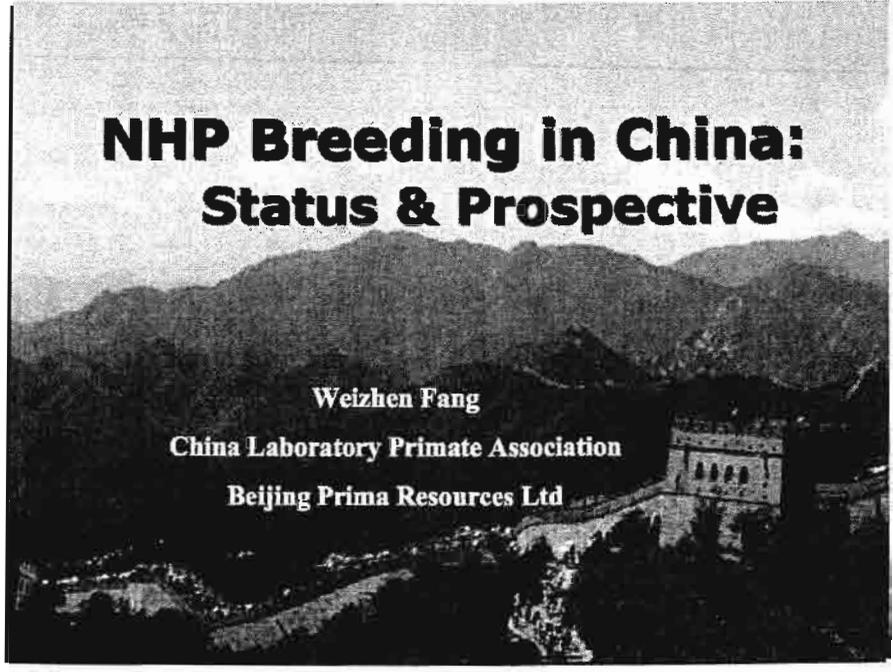
Enrichment Devices
Products designed and developed specifically to enrich the lives of captive nonhuman primates.
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Attention to psychological well-being of nonhuman primates is built right into our housing...
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Handling Equipment
Our products are often imitated but, when it comes to the handling captive nonhuman primates, PPI...
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Panther Tracks Learning Center
The best research starts with the best training. PPI is enhancing worldwide biomedical research programs through effective educational and training programs at P.T.C.
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NHP Breeding in China: Status & Prospective

Weizhen Fang

China Laboratory Primate Association

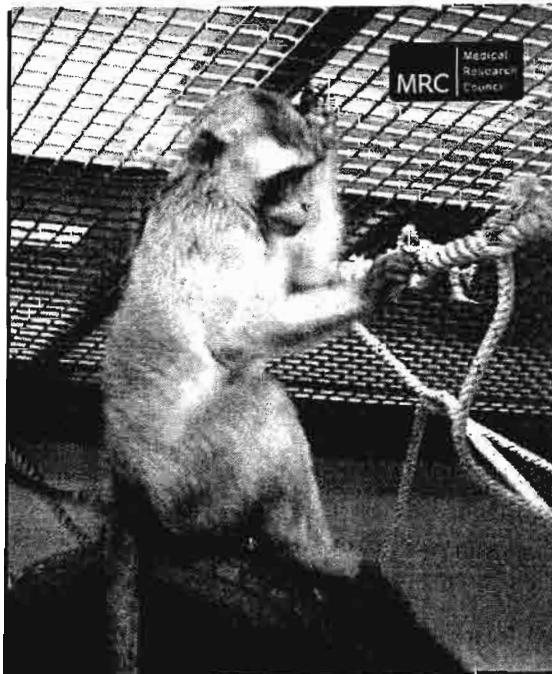
Beijing Prima Resources Ltd

Survey in 2007

- Survey on 31 breeding facilities, by CLPA in May, 2007.
- Building: over 1,000,000m² , increased many folds in four years.
- Total number: About 150,000, including 120,000 Cynos, 30,000 Rhesus.
- Total Export in 2006; about 19,000 .



Centre for macaque
UK



Porton Dawn
UK

SCHIZOPHRENIA
ALZHEIMER'S DISEASE
STROKE
PRESENILE DEMENTIA
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HEPATIC
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HEPATIC
PARKINSON'S
ALZHEIMER'S
PARKINSON'S DISEASE
SCHIZO

PRIMATES IN MEDICAL RESEARCH

The research environment
Primates are highly intelligent, sociable and energetic animals. They need a stimulating, varied environment that reflects aspects of their natural habitat. This includes being housed in compatible sexes or groups, with plenty of vertical space and options for climbing and swinging and increased access to trees where they can play for food and social interaction. They should also be allowed to forage for their food, which encourages their problem-solving skills as this is an important daily activity in the wild.

Familiarisation with humans
The way in which primates interact with humans has a major impact on their welfare. Increasingly, animal care staff are using primates, such as vervet monkeys, to produce changes in behaviour and to measure their fear or stress. As well as being more humane, this can also reduce variability in research results and therefore increase their reliability.

Veterinary care
All primates require the same level of care and attention as other animals. All primates must be held in a clean, well-ventilated, and fully equipped laboratory. All primates must receive painkillers and other care for as long as is necessary to prevent discomfort and hasten recovery.

What happens to animals after the research?
The majority of primates used in research are eventually placed in sanctuaries where they can live out their lives in a natural environment. Placement in an alternative environment for some animals, if they are healthy and a suitable environment exists.

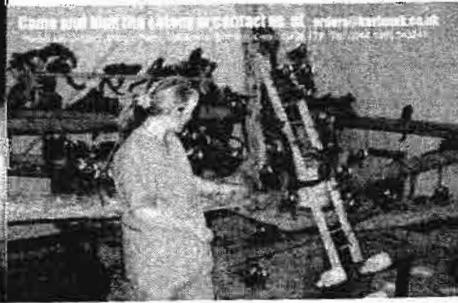


Harlan
UK

Harlan

The Harlan marmoset colony was established in 1979 from a selection of primates within the UK primate research industry. The colony was maintained as a closed colony from 1980 - 1987 when additional laboratory bred stock from a major UK Primate supplier (Cephalopithecus) were introduced to expand its capacity for the colony as it developed as an established population with a strong genetic base. To enhance animal welfare and to maintain colony support a long-term of collaborative relationship has been successfully introduced.

Large breeding cages provide large groups with up to three levels of climbing. On average, 10000 animals are housed in single sex groups in large comfortable housing rooms with a fully equipped environment. Animals are housed in groups together with an abundance of delivery to enhance compatibility. Delivery is either by hand in the UK or Harlan air conditioned animals or by air transport (air) in purpose-built IATA approved containers.



B & K
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the B&K name is synonymous with quality breeding for medical research. Under the (Procedures) Act 1986, B&K Universal are a breeding and supplying establishment and through a 'Culture of care' we exceed the down by the Home Office.

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Biomedical Primate Research Centre
COMMITTED TO THE BEST RESEARCH AND ALTERNATIVES

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Animal Welfare / Welfare Plans

Certain aspects of animal welfare at BPRC have been limited in the past due to limited amounts of room and the outdated nature of the facilities. This meant that many animals were housed alone under circumstances that were far from optimal. Monkeys are social animals, and should be housed in social groups wherever possible. In 1984 a start was made to improve this situation by initiating social breeding-groups in outside enclosures (the "meadows"). This style of housing has been continued since 1984 but due to very limited financial means it was not possible to provide this for all animals.

This has long been a cause for concern at the BPRC; in this period larger cages have also been built or brought in to allow social housing of more animals. However the financial constraints meant that a large proportion of the colony remained in suboptimal conditions.



Research facility

The situation changed dramatically when the Ministry of Education, Culture and Sciences (O, C en W) decided that plans for better housing of the animals by the BPRC-management were justified. This, together with enquiries from different animal welfare organizations led to an agreement whereby the Ministry is investing substantial amounts of money to permit the rebuilding of the entire BPRC facility to bring it up to the highest modern animal welfare standards. In addition the Stichting Vereniging ter Bescherming van Dieren (The Sophia Association for the Protection of Animals) provided important

financial support stating that 'as long as society decides that animal experiments for biomedical research are necessary, the animals should be housed under the most optimal conditions'. This support to improve the conditions of animal welfare has been gratefully acknowledged by BPRC.

DPZ Germany



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About us

The German Primate Centre (DPZ, founded in 1977) is a non-profit independent research and service institute. It is a member of the Leibniz Association and funded by the federal government and by the states of Germany. In addition, about 40 % of the total budget of 15 million Euro comes from grants.

The functions and services of the DPZ concentrate on biological and biomedical research on and with primates and include the study and maintenance of the ranging primate populations and improvements in husbandry of primates in human care.

The DPZ's mission is to serve as a center of excellence for research with primates and as a service and competence center for those institutions in Germany and abroad that house primates and/or do primate-related research (e.g. academic laboratories and zoological gardens). The center is organized in three sections: Organismic Primate Biology, Neurosciences and Infection Research.

The DPZ is closely cooperating with the University of Göttingen and the local Max-Planck Institute. Heads of departments hold professorship at the University of Göttingen or at the Faculty of Veterinary Medicine in Hannover.

Since the DPZ is unique in Germany and only one similar institute exists in Europe, the Center is of international importance.

DPZ Europe



last update February 2007

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37077 Göttingen
Germany
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Fax: ++49-551-3851-228
www.dpz.gwdg.de

The Shareholders are the Federal Republic of Germany and the State of Lower Saxony.

The Supervisory Board is elected by the share-holders and consists of nine members. It is chaired by the State of Lower Saxony.

The Scientific Advisory Board advises the DPZ and the Supervisory Board in matters of the long-term structure and research projects. It currently consists of ten national and international members.

Management
Prof. Dr. Stefan Treue, Director
stue@dpz.gwdg.de

Michael Lammel, administrative manager
m.lammel@dpz.gwdg.de

ULP
France



2002 / 2003

Centre de Primatologie

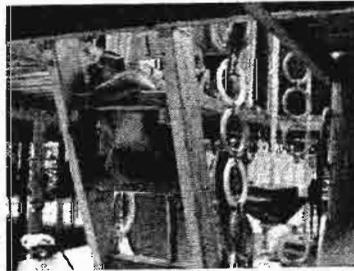
Nicolae Harnescu
Docteur de l'ULP
Drochia

Famula Wanzel
Docteur Veterinar
Drochia Ajanta

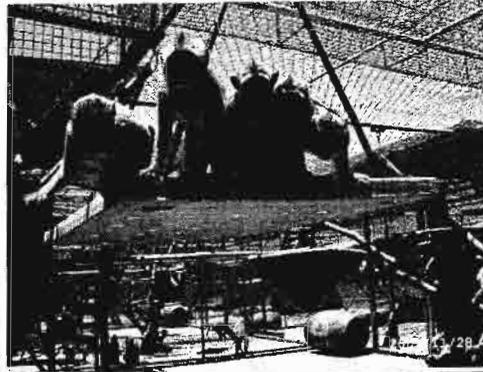
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Mailingadresse: Centre de Primatologie
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primatologie@ulp.fr



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PHILIPPINE PRIMATE CENTER



The World's Largest

Miqaca Fascicularis Captiva Breeding Colony

Primates for Biomedicine and Conservation



SMI
Sweden



BioPRIM
France



Chers collègues, chers amis,

Les agréments de BioPRIM viennent d'être confirmés :

- Autorisation d'ouverture d'établissement du 29 novembre 2001
- Déclaration d'établissement et livraison et fournisseur d'anténaux pour l'expérimentation, N°10705
- Certificat de capital, N° 67-001

Nous tenons vivement à remercier tous ceux grâce à qui, l'intérêt, la qualité et le professionnalisme de BioPRIM ont été reconnus et permet aujourd'hui de mettre à votre disposition ce Centre de Primatologie.

Dans l'attente de nos collaborations futures, recevez chers collègues et amis toute notre gratitude.

Eric Andrieu
Gérant de BioPRIM

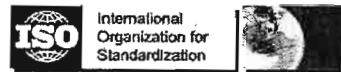
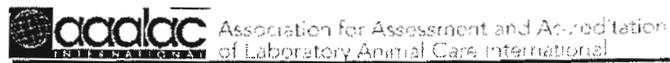


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Leading in safety & security

- ***Our animal security systems and SOP's for the prevention of animal escape, form part of our internationally accredited Quality System and are a fundamental part of our operations.***

We have AAALAC accreditation and ISO certification



Animal Security systems

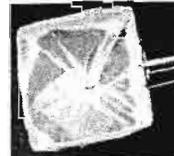
- Our animal security systems for the prevention of animal escape operate on a number of levels:
- Staff training and inspections
- Cage design and engineering - hurricane proof cages.
- Provision of appropriate security equipment and systems

Safety and Security measures

- Training of workers on security SOPs.
- Daily cage inspection and supervision.



- Handling equipment
- Electric fencing around the farm.

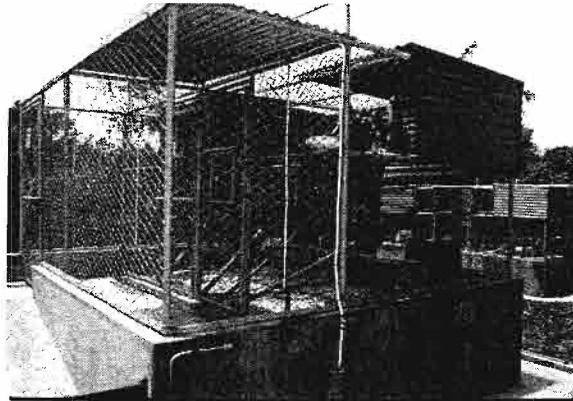


- Double door system on all cages.
- Monkey proof latches and locks on all doors.



Hurricane Proof Cages

- Cages especially designed and engineered to be cyclone proof.



Bioculture-PR commitment

- ***Provide the world's highest standards in terms of animal security and welfare.***

Bioculture Puerto Rico Inc.



Phone = 7875909620

Email: Owen Griffith's - Owen@bcmpr.com

Email: Bushmitz Moshe - moshe@bcmpr.com



Joseph W. LaPlume
Charles River Laboratories
251 Ballardvale Street
Wilmington, MA 01887
(781) 222-6230
joseph.laplume@crl.com

May 2, 2008

Dr. James Perez, Ph.D.
BioScience Consultant-Life Sciences
Puerto Rico Industrial Development Company

Dear Dr. Perez:

Charles River Laboratories, Inc. (CRL) has successfully distributed Mauritian origin monkeys (NHP) from Bioculture (Mauritius) (BCM) since 1989. BCM is a company which is well regarded in the industry and which has always focused on good animal husbandry with a particular emphasis on animal welfare. BCM has met the required international standards and has achieved AALLAC accreditation.

It is recognized that the clean profile of Mauritius NHPs makes them important to the biomedical research community.

While we cannot predict the future, the presence of such a colony in Puerto Rico could provide Puerto Rico with a valuable resource. CRL is looking forward to continue to work with BCM to provide high quality NHPs to the biomedical research market.

Very truly yours,

Joseph William LaPlume
Senior Corporate Counsel

JWL/acg

Points for the Amendment

- 1) An explicit statement to the effect that the sole purpose of importing the animals to Puerto Rico is their eventual use in scientific investigation, even though Bioculture will not be doing the investigation itself.
- 2) A brief description of the types of investigation that Bioculture's clients (regardless of where the investigation will take place) will perform with the animals. Needless to say, no trade secrets/particular methodology needs to be included. For example: "ACME Medical Laboratories, Inc. will use the animals to harvest tissue to test antibiotic resistance", etc. For each activity, the Department will also need a short description of the benefits expected from the investigation. For example: "the investigation to be conducted by ACME will result in the discovery/development/approval of new antibiotics for human/veterinarian use".
- 3) Amount, age and gender of the animals to be imported to start the colony in Guayama.
- 4) A statement of benefits to the species. Even though we believe that this particular item is inapplicable to our activities, we can include a brief statement as to the humane nature of the conditions of confinement and how the center will definitely promote the well being of the animals while they are there.
- 5) Evidence of applications for permits with other regulatory agencies, with particular emphasis on USDA.
- 6) Evidence from the UPR , as well as from any other university, research institution/company, that establish Bioculture's good standing in the scientific community and its satisfactory operations elsewhere in the world. It would also be helpful to include the UPR's invitation to Moshe to lecture as a visiting professor.

Info requested as amendment to the Animal Importation Permit for Bioculture (Puerto Rico) Inc.

1. **Reason for Import.** The purpose of importing animals into Puerto Rico is for their ultimate use for scientific purposes in Biomedical Research (even though Bioculture is not carrying out the research itself). Bioculture will be importing animals to Puerto Rico to supply to companies engaged in Biomedical Research or to be used as breeders, held under specific conditions, with their offspring being provided for use by companies engaged in Biomedical Research in the USA and overseas and ultimately in Puerto Rico itself.

2. **Types of Scientific investigations that will be carried out by Bioculture's clients using Bioculture's animals .** The main purpose of the Bioculture project will be to hold; breed and rear monkeys for eventual use in Scientific Research. The clients for these animals will be Pharmaceutical companies, CRO's, Universities, NIH Research Centers and private Research Centers. The monkeys will be exported mostly to the continental USA or used in Puerto Rico if needed by universities or research institutes. There may be some requirements to also export animals to Europe.

Mauritian origin monkeys enjoys definite advantages as they are naturally SPF, being naturally free of a number of significant viruses. Thus Mauritian origin monkeys are free of Herpes B virus, STLV1 SRV. (see attached file - "*Importance of Primates to Biomedical Research*")

We have 4 types of clients :

- a. **Pharmaceutical companies & CRO's** - they are using our primates for drug development, safety testing, toxicology and vaccines. This is done in different fields such as reproduction, neurology, infectious diseases and others.
- b. **Academia** - includes Primate Centers and Universities - they are doing basic and translation scientific research - like AIDS, Parkinson, MS , Alzheimer and other fields.
- c. **Regulatory agencies** - like NIBSC ,FDA,CDC ,WHO ,NIH or government regulatory agencies - like the Sweden Infectious Diseases Institute; the Dutch Vaccine Institute and others.
- d. **Biotechnology companies** - they usually develop instruments like pacemakers, deep brain stimulation, insulin pumps or drug delivery systems.
- e. Our client list includes (confidential):

Bayer - Germany
Covance - Germany
Covance - UK
Charles River - Scotland
FARMACIA - Italy
Novartis - Switzerland
LCG Bioscience - Italy
GSK - Belgium
Nerviano – Italy
Aventis Sanofi – France
Astra Zeneka –UK
Charles River - USA
Charles River – Canada
Pfizer -USA
Roche- USA
SNBL - USA

Maccine - Singapore
Teva - Israel
Government of UK
Government of Sweden SMI
Government of Canada
NIBSC - UK
Porton Dawn -UK
Hebrew University - Israel
Bar Ilan University- Israel
Technion University- Israel
Tel Aviv University- Israel
Weitzman Institute- Israel
University of London
Oxford University
UPR - PR
NIH Research Centers
Wisconsin - NIH primate center

3. Amount, age and gender of the animals to be imported to start the colony in Guayama.

The age range will be from 2.5 - 5 years

The gender will be mostly females and small number of males.

1st phase we plan to import up to 1000 animals

2nd phase we plan to import up to 1000 animals

3rd phase we plan to import up to 1000 animals

4th phase we plan to import up to 1000 animals

5th phase we plan to import up to 500 animals

The total importation of breeders will be up to 4500 animals within 5 years

4. Benefit to the species.

Monkeys are a vital input for Scientific Research. By breeding monkeys for research, Bioculture is providing animals to the scientific community in a humane and sustainable way, thus reducing pressure on native populations in South East Asia. Further more breeding animals in Puerto Rico will mean that the travel time for monkeys used in the USA will be dramatically reduced as opposed to animals imported from Mauritius and this will provide a clear welfare benefit.

5. Evidence of applications for permits with other regulatory agencies, with particular emphasis on USDA.

We have already met twice with the USDA inspector that will be supervising Bioculture in Puerto Rico. He has looked at the plans and our program and has no comments at this stage. Attached is draft of the USDA application. USDA will come to the site 45 days before the importation - without their approval Bioculture cannot bring the animals. The USDA is the Federal supervisory agency of all primate facilities in PR including the UPR in Sabana Seca and Cayo Santiago.

6. Bioculture's good standing in the scientific community and its satisfactory operations elsewhere in the world.

Bioculture has been operating since 1985 at the highest possible standard. This is evidenced by the various Industry accreditations/certifications we have as well as our linkages with other organizations.

- a. Bioculture is AAALAC accredited - in the field of Biomedical Research and in the fields of the use/production of lab animals, this accreditation is the highest standard and most universities and research companies have this accreditation (see attached "AAALAC CERTIFICATE").
- b. Bioculture is ISO 9001 certified since 1998.

- c. Bioculture has UK Home Office approval since 1995 (last renewal in 2009).
 - d. Consortium with UPR Primate Center - see the goal declaration (IRPC)
 - e. Cooperation project on Stem Cell research with UPR Primate Center - see a presentation on the project done in a welfare meeting in London in September 2009 . Note this is also an NIH funded project.(see attached file - "*Stem cell presentation- UPR*")
 - f. See attached letter from Dr. Carol Brenner from April 2008 about the use of our animals for the ES production in the UPR center - ("Cyno ES cells - April 14th 2008")
 - g. See Prof Barry Bavister letter to PRIDCO in March 2008 on the importance of use of our animals for the scientific development in PR and UPR (Bavister letter to Perez).
 - h. See the attached article : "*The research on primate embryonic stem cells is crucial for developing human therapies.* "
 - i. See attached article: "*Stem Cells, Regenerative Medicine, and Animal Models of Disease*".
 - j. Professor Moshe Bushnitz nomination by the UPR medical school.(see attached nomination) - Bioculture and UPR are clearly named in the nomination.
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Bioculture Mauritius Ltd.

*Sainte-ville, Riviere des Anguilles
Mauritius*

February 17, 2006

Production of Cynomolgus Monkey Embryonic Stem Cells

INTRODUCTION

Cynomolgus monkeys from Mauritius possess an extremely high tissue compatibility with their population, greatly reducing the possibility of graft vs. host rejection in tissue/organ transplantation. As Krebs et al. (2005) have reported, "Animals from Mauritius may be particularly valuable because >50% of these Cynomolgus macaques share the MHC class I allele combination *Mafa-B*430101*, *Mafa-B*440101*, and *Mafa-B*460101*". Therefore, tissues from these animals would be valuable for transplantation work in a non-human primate model.

Embryonic stem cells (ESCs) are pluripotent cells that proliferate rapidly in culture and under the proper conditions, can maintain pluripotency (undifferentiated state) indefinitely, or may be stimulated to differentiate into specific cell types (reviewed by Hoffman and Carpenter 2005). This ability to differentiate into any cell type raises the possibility that ESCs can be used to replace tissues or organs damaged by disease. Therefore, ESC research holds immense promise for treating diseases such as Parkinson's disease, juvenile onset diabetes, heart disease and leukemia. Realizing this promise, however, will require the use of non-human primate models to test the efficacy and safety of ESC-based transplantation therapies.

Given the challenges of tissue rejection when cells are transplanted into a different individual, new strategies are being devised to produce ESCs from the patient to be treated through human cloning, induction of somatic cells (e.g., skin cells) to become ESC, and other approaches. It would be a great advantage to devise a non-human primate model to pilot ESC-based transplantation therapies without having to generate a new ESC line from every animal to be tested. The Mauritian cynomolgus macaques could provide such an experimental model. An ESC line derived from these animals could be transplanted into any Mauritian macaque, since they would share the key histocompatibility markers. Therefore we propose to derive ESC lines from Mauritian cynomolgus macaque embryos produce by in vitro fertilization at the Caribbean Primate Research Center in Sabana Seca, Puerto Rico.

GOALS

ESC will be derived from Mauritian cynomolgus macaque embryos through studies conducted in two phases.

Phase 1. Establish embryo production from Mauritian cynomolgus macaques. The purpose would be to evaluate and optimize in vitro embryo production and culture in the cynomolgus monkeys using procedures long established by our laboratory in the rhesus macaque. This phase will require approximately 15 animals and could be completed between June and October, when work on rhesus macaque embryology ceases. Cost for this phase would be modest.

Specifics of Projects in Phase I

1. Are Mauritian cynomolgus macaque embryos, produced by in vitro fertilization, chromosomally normal?
2. Do Mauritian cynomolgus macaque embryos, produced by in vitro fertilization, have high quality blastocysts for stem cell production.

Budget needs are for supplies:

The supplies needs for this budget are chromosomal probes, antibodies, disposable tissue culture, personnel, veterinarian, hormones, shipping costs, travel. I am projecting costs for 10-20 cycles of monkey IVF. Tiffini Gibson, an experienced primate embryologist from my laboratory will be performing the IVF.

1. Personnel/ monkey IVF cycle	\$10,000
2. Veterinarian fees/monkey IVF cycle	\$10,000
3. Hormones/ monkey IVF cycle	\$10,000
4. Culture media and supplies	\$ 5,000
5. Shipping embryos to lab for analysis	\$ 2000
6. Chromosomal analysis	\$ 2500
7. Stem cell quality analysis	\$ 2500
8. Travel for Tiffini Gibson	\$ 2500

Total costs: \$50,000

Phase 2. Derive ESC from Mauritian cynomolgus macaque embryos.

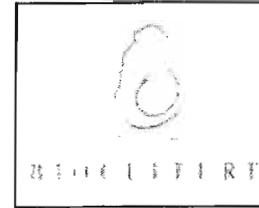
The purpose of this phase would be to generate large numbers of embryos for derivation of ESCs from Mauritian cynomolgus macaques. This phase will require approximately 50 animals and will require approximately one year to complete.

Cost for this phase would be higher.
To be determined.

Reference:

Hoffman LM, Carpenter MK (2005) Characterization and culture of human embryonic stem cells. *Nature Biotechnology*, 23: 699-708.

Krebs KC, Jin ZY, Rudersdorf R, Hughes AL, O'Connor DH (2005) Unusually High Frequency MHC Class I Alleles in Mauritian Origin Cynomolgus Macaques. *The Journal of Immunology*, 175: 5230-5239.



International Primate Research Consortium - IPRC

The IPRC is a Research Consortium between CPRC and BCMPR, established to enhance and facilitate collaborative research programs of the two Centers with academic institutions and the private sector.

The primary objective of IPRC is to promote translational research projects of scientific relevance that make use of Specific Pathogen Free (SPF) Non Human Primates (Cynomologus and Rhesus Macaques).

These research areas include: Stem Cell Research, Assisted Reproduction technologies, Diabetes, Cardiovascular Diseases, Vaccine Development, Microbial Pathogenesis, Genetics and Immunology.

Both Centers are leading suppliers of SPF macaques that are used worldwide in biomedical and behavioral research projects.

Both centers will share knowledge, expertise, data and resources .



Oficina
del Rector

Universidad de Puerto Rico
Recinto de Ciencias Médicas

10 de febrero de 2009

CARTA CIRCULAR 2008-2009

Dr. Walter Frontera
Dr. Walter Silva


José R. Carlo, MD
Rector

NOMBRAMIENTO PROFESOR ADJUNTO

Luego de evaluar las recomendaciones y credenciales sometidas por el Decano de la Escuela de Medicina, y en concordancia con la Certificación Número 024 (1996-97) de la Junta de Síndicos de la Universidad de Puerto Rico, YO, Dr. José R. Carlo Izquierdo, Rector del Recinto de Ciencias Médicas de la Universidad de Puerto Rico, otorgo el nombramiento de Profesor Adjunto al **Dr. Bushnitz Mark Moshe**. El nombramiento estará bajo la Escuela de Medicina adscrito al Decanato de Medicina.

El doctor Moshe posee Doctorado en Medicina Veterinaria de "Universita di Bologna" en Italia (1982) con una especialidad en "Laboratory Animal Medicine" en "Hebrew University" en Jerusalem, Israel (2006). El doctor Moshe es el fundador y el Director de la Compañía BFC localizada en Israel. El doctor Moshe es también consultor en manejo, cuido y bienestar de los primates de la Universidad de Bar Ilan, de la Universidad Hebrea en Israel, de la Compañía BioCulture en Mauritius y de nuestro Centro de Primates. Los principales intereses del Doctor Moshe son el comportamiento animal e interacción social. Ha sido instrumental en recomendaciones para nuestro plan estratégico en el cuido, nutrición y bienestar de la colonia en Sabana Seca y en los diseños de estructuras para maximizar el uso de los terrenos del Centro. El seguirá dando recomendaciones para el manejo de la colonia, y proveerá seminarios al personal en éste y otros tópicos de cuido veterinario. El doctor Moshe es un experto reconocido por la comunidad científica en este campo de la Primatología.

Según la disponibilidad de Fondos, su nombramiento podrá estar activo por cinco (5) años. Todas las disposiciones de la Certificación Número 024 (1S 1996-97) estarán vigentes.

Y PARA QUE ASÍ CONSTE, expido la presente Carta Circular en San Juan, Puerto Rico, hoy 10 de febrero de 2009.

jpo

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Primera con
Igualdad
de Oportunidad
en el Empleo
M.M.V.I

April 17, 2008

To whom it may concern.

I am the head of the Experimental surgery and Laboratory Animal Unit at the faculty of Medicine – Technion, IIT (Israel Institute of Technology).

I have over 20 years of experience working with R&D for large pharmaceutical companies and biomedical startup companies developing medical devices in the field of cardiology.

Many species of animals have been used in our studies during these years, including SPF Cynomolgus monkeys of Mauritian origin. These animals possess extremely high tissue compatibility within their population, greatly reducing the possibility of graft vs. host rejection in tissue/organ transplantation.

A good source of SPF animals combined with government support for biomedical and pharmaceutical research and development will be great interest to CROs, biomedical startup companies and drug development in Puerto Rico.

Knowing personally the people involved, I am sure this project of Mauritian breeding in PR will give PR an advantage in attracting such companies to the island.

Kind Regards,

Rona Shofti
Head Experimental Surgery & Laboratory Animal Unit



Research on Primate Embryonic Stem Cells Is Crucial For Developing Human Therapies According to Cloning and Stem Cells

LARCHMONT, N.Y. -- Studying the embryonic stem (ES) cells of primates in parallel with ongoing research on human ES cells will accelerate the knowledge gained, improve techniques for working with ES cells, and maximize the potential for developing powerful new therapies to treat human degenerative diseases such as diabetes and Parkinson's and Alzheimer's disease, according to a provocative report to be published in the Summer 2005 (Volume 7, Number 2) issue of *Cloning and Stem Cells*, a peer-reviewed journal published by Mary Ann Liebert, Inc. The paper was published online ahead of print and is available free online at www.liebertpub.com/clo. The great promise of embryonic stem cells for treating a range of human diseases will only be realized through combined research on human ES cells and studies in animal models. Intensive research and protocol development using ES cells derived from rhesus monkey embryos would greatly contribute to and accelerate the optimization of techniques for transforming human stem cells into safe and functional cells, tissues, and organs for replacement therapy. Commonly studied mouse ES cells are not very good models for human ES cells, and research on mouse cells makes a limited contribution to our understanding and ability to work with human ES cells, contend Barry Bavister, Ph.D., Don Wolf, Ph.D., and Carol Brenner, Ph.D., of the University of New Orleans and the Oregon National Primate Research Center, and authors of the paper entitled, "Challenges of Primate Embryonic Stem Cell Research." In this Opinion Piece, the authors make a powerful case for additional research with non-human primate embryo stem cells in parallel with those on human embryo stem and adult stem cells," says Ian Wilmut, Ph.D., Editor-in-Chief of *Cloning and Stem Cells* and Head of the Department of Gene Expression and Development at the Roslin Institute. "As the authors point out, working with an experimental animal is the only way to gain all the information needed about the biology of normal stem cells in a primate. As all human embryo stem cells are obtained from embryos produced during in vitro fertilization procedures, it is possible that they are all affected by the period of embryo culture," Wilmut says. "The present culture regimes are known to cause changes in the embryos that may persist in embryo stem cells. By contrast, rhesus monkey cells have been derived from embryos recovered from mated donors. Furthermore, it would be possible to assess the use of cells to treat disease in an animal model with similar physiology to humans and a longer lifespan than the mouse." *Cloning and Stem Cells* is an authoritative peer-reviewed journal published quarterly in print and online that focuses on understanding developmental plasticity and defining the molecular mechanisms that regulate differentiation or dedifferentiation of nuclei and cells. Tables of contents and a free sample issue may be viewed online at www.liebertpub.com/clo. Mary Ann Liebert, Inc., is a privately held, fully integrated media company known for establishing authoritative peer-reviewed journals in many promising areas of science and biomedical research, including Human Gene Therapy, Stem Cells and Development, and Tissue Engineering. Its biotechnology trade magazine, *Genetic Engineering News (GEN)*, was the first in its field and is today the industry's most widely read publication worldwide. A complete list of the firm's 60 journals, books, and newsmagazines is available at www.liebertpub.com.

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Barry D. Bavister, Ph.D.

Adjunct Clinical Professor, Department of Obstetrics & Gynecology
Wayne State University Medical School

Adjunct Professor, University of Puerto Rico
Scientific Co-Director of Reproduction Research
Caribbean Primate Research Center, Puerto Rico

Wednesday, May 12, 2010

James Pérez, Ph.D.
BioScience Consultant - Life Sciences
Puerto Rico Industrial Development Company
355 F.D. Roosevelt Avenue
Hato Rey, Puerto Rico 00936-2350

Dear James,

Here is information to support my enthusiastic response to the proposal to bring cynomolgus monkeys to Puerto Rico.

1. My credentials

I obtained my PhD in Reproductive Physiology from the University of Cambridge in 1972. From 1978-2000 I was a professor at the University of Wisconsin-Madison, and from 2000-2008 I have been a tenured professor at the University of New Orleans. (See my attached Biosketch). I am leaving UNO in May to become a full-time consultant for the UPR-WSU consortium (see below). While I was a graduate student in Cambridge, I helped develop the first human in vitro fertilization process, and in Madison my lab produced the first IVF (rhesus) monkey. I have worked with rhesus monkeys since 1979. During my career I have published >200 refereed articles and book chapters and published three books, all on the topics of mammalian gamete and embryo biology. Many of these articles are about research using rhesus monkeys. My work in recent years has focused on the extension of embryo development into embryonic stem (ES) cells. *

For the past three years, I have been involved with the Caribbean Primate Research Center in Puerto Rico. This Center is small compared with the 8 federally-supported US mainland National Primate Research Centers, but it has a huge advantage: a relative abundance of rhesus monkeys that are not assigned to AIDS research or other major research programs, in contrast to the NPRCs. I have established a small embryology laboratory at CPRC that conducts research relevant to human infertility, and produces preimplantation embryos (essentially, fertilized eggs) that we send to WSU for ES cell research. The CPRC has been very generous in providing animal resources for this program and is enthusiastic about supporting and expanding our research efforts.

2. Macaque monkeys are excellent as models for human health research, including infertility, and for ES cell research. Macaques share many characteristics with humans, including their reproductive endocrinology, embryology, pregnancy, and ES cell characteristics. The macaques are menstrual-cycling monkeys and have singleton births. The so-called New World monkeys, such as marmosets, are smaller and easier to house but their reproductive characteristics are considerably different from humans, e.g., they have estrous cycles not menstrual cycles.

The rhesus monkey (*Macaca mulatta*) has become the de facto model for many kinds of studies related to human health, largely because of the huge amount of research data on this species. My lab has used this species extensively for almost 30 years. However, there are some drawbacks to using rhesus monkeys for studies related to human reproduction. These are rather large, aggressive animals, and they carry a variety of viruses, one of which, Herpes-B, can be lethal to humans. There have been a few deaths among researchers following contamination with fluids from rhesus monkeys and lack of immediate treatment. However, the major rate-limiting factor with using rhesus monkeys is their seasonal reproductive behavior: from roughly May through August the animals stop cycling, so for these four months every year we cannot use the animals for reproductive or for ES cell research. This represents a huge loss of productivity, and inefficient use of grant funds.

Some reproductive studies have used cynomolgus monkeys (*Macaca fascicularis*) instead of rhesus monkeys. “Cynos” as they are often called share many of the desirable characteristics of rhesus monkeys: their reproductive cycles and endocrinology, and embryonic development, are very similar if not identical. However, cynos have some very beneficial differences compared with rhesus monkeys: they are non-seasonal, meaning that they cycle year-round. Using cynos for reproductive and ES cell research would effectively increase our productivity by 50% (12 months per year instead of 8). This would have a major impact on our ability to generate data, publish research, and obtain more federal funding. I propose to augment our group’s research efforts using cynos, emphasizing their use during the summer months when the rhesus monkeys are unusable. In addition to this advantage for our research, cynos do not carry Herpes-B virus, which would greatly reduce the danger to the public from any escaped animals, or harbor several other viruses that could negatively affect our research. For example, no-one has studied the possible effects of CMV or “foamy” viruses on ES cells produced from rhesus monkey embryos. Our expectations to develop a major research and commercial program based on rhesus ES cells could be damaged if these viruses were found alter the properties of ES cells; we could use SPF rhesus monkeys instead but there is already a huge demand for these animals for biomedical research. In contrast, cynos are less in demand because they are not as useful for AIDS research.

Cynos are also smaller and less aggressive than rhesus monkeys, which makes them easier to house and handle. Moreover, because the CPRC supports itself in part by selling rhesus monkeys to the NPRCs, reducing our group’s needs for rhesus by replacing them with cynos would help the CPRC financially.

In summary, the availability of cynos at the CPRC, and the presence of a cyno breeding colony elsewhere in Puerto Rico to produce the animals, would be a huge benefit to our consortium’s research efforts and strategic plans.

3. In 2007, my colleagues and I began to establish a consortium of researchers between UPR/CPRC and the Department of Obstetrics and Gynecology at Wayne State University in Detroit. This consortium presently numbers about seven primary researchers and another 20 secondary colleagues. The Medical School at WSU is one of the nation’s best and the Ob/Gyn Department has an exceptional record of federal funding for basic and clinical research. I hold adjunct professor

appointments at both institutions and my major function is to foster the UPR/WSU research relationship.

A major interest, and the primary basis for our collaborative research program, is nonhuman primate (NHP) embryology and ES cell research. The NIH has (belatedly) recognized the central need for NHP reproductive and ES cell research, after decades of supporting mostly mouse studies that have not resulted in any significant improvement in clinical treatments for human infertility. Moreover, although mouse ES cell research has produced some useful insights, it is now clear that these cells differ significantly from primate (NHP and human) ES cells, so that the latter must now be pursued more aggressively. For a variety of reasons – ethical, practical and scientific – NHP ES cells have become paramount players in the national strategy for translational research aimed at transplantation of in vitro derived tissues and organs. Because no new human ES cell lines can be created using federal funds, and existing cell lines are senescent and contaminated with mouse feeder cell components, new NHP ES cell lines are filling the void. Even if the US government eventually relents and allows new human ES cell lines to be created for research, using these cells for commercial purposes is still objectionable to many people.

4. Strategic plans

We are at the beginning of our UPR/WSU consortium relationship. During 2008, WSU will provide some funds to expand the CPRC facilities (mainly office space). CPRC will increase consignments of rhesus monkey embryos to WSU to support ES cell and related research at WSU, along with continuing a small embryology research program at CPRC including production of high quality embryos obtained from mated animals. WSU is writing several NIH grants involving UPR to expand and support our collaborations. One of them, due this summer for 2009 funding, is an interactive Minority Cooperative Program (U54) that will support training and research of Puerto Rican students at WSU as well as research activities at both institutions. At least six other NIH grants are in preparation or planned for 2008 submission.

In the medium term (2008-2011), WSU will help UPR to develop its research capabilities, with projects by WSU scientists and clinicians using the CPRC resources; some components will be on site' at CPRC. There will be further expansion of animal and physical resources at CPRC. Addition of cynos, for reasons mentioned above, would be a great help to our efforts.

In 2009, I will help to write a substantial increase in the CPRC base grant (from NIH/NCRR) to support the embryology/ES cell program. WSU will use CPRC embryos to develop novel ES cell technology and help establish an ES cell laboratory at UPR (in Dr. Kraiselburd's lab space). This will require high level training of Puerto Rican technical and scientific staff to operate and expand the lab's capabilities.

I would propose to approach drug companies to use our monkey ES cell lines generated at UPR for drug discovery and development. As mentioned above (and see attached article) NHP ES cells have some fundamental advantages over human ES cells, and could be used without ethical objections. I need help with this venture! However, I believe there is much potential for employment of Puerto Rican personnel to perform NHP ES cell research and to produce ES cell lines for commercial purposes. Because ES cell lines age and become unusable after some months, it is necessary to replace the cells periodically, thus supporting an ongoing cell production facility. Also there is a need to monitor the cells for normality and pluripotency, which necessitates further lab-based activities. Possibly, UPR could become a national or an international center for production, testing and research of NHP ES cells. We might possibly attract drug companies to establish facilities in Puerto Rico to help these research and commercial endeavors.

The above ideas were originally based on availability of only rhesus monkey embryos to make ES cell lines. However, if cyno monkeys were also available, our ES cell productivity could increase by as much as 50%, for reasons discussed above. The consequent impact on the practical implications for Puerto Rico could be dramatic. Imagine if cynos were not available: each summer for 4 months, there would be no embryo or ES cell production, so all these plans would come to a halt until October (September is the month that rhesus monkeys start to cycle again and it usually takes the whole month to gear up again).

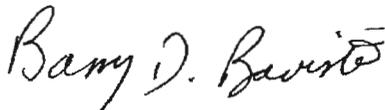
In the longer term (2011-2014), using novel ES cell technology developed by WSU, UPR will establish and operate its own ES cell laboratory to perform translational research (tissue and organ transplantation) and to serve commercial (drug company) interests as suggested above. We anticipate that UPR/CPRC will become known internationally as a center for research and training in monkey embryology and ES cells; both areas are highly translatable to human clinical health and medical interventions.

5. Summary

Without cynos being available in Puerto Rico, the UPR/WSU consortium plans as outlined above will go ahead. These activities will involve Puerto Rican scientists, clinicians, students and technical staff. We earnestly desire to engage island personnel in our plans. An integral component of our plans will be opportunities for scientific and clinical research collaborations and for UPR student training at WSU. However, the lack of rhesus embryo materials during 4 months every summer (5 months counting the September "startup" period) is a serious impediment. If cynos could be used in addition to rhesus monkeys, our research, training and potential commercial activities would be substantially accelerated.

I hope this information is helpful for your report. Please do not hesitate to contact me if you need additional information.

Best wishes,



Barry Bavister, Ph.D.

cc: Edmundo Kraiselburd, Ph.D.

attachments: article on NHP stem cells; Bavister NIH Biosketch

<p style="text-align: center;">Barry Bavister, Ph.D. 4505 Dryades Street, New Orleans, LA 70115, USA Tel: 504 874 1084 • FAX: 504 897 2143 E-mail: bbavister@rcm.upr.edu • Website: www.barrybavister.com</p>
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ORIGINAL ARTICLE

Genetic diversity of longtail macaques (*Macaca fascicularis*) on the island of Mauritius: an assessment of nuclear and mitochondrial DNA polymorphisms

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Keywords

bottleneck – introduction – microsatellite – mtDNA

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Accepted February 22, 2007.

Abstract

Background Individuals from an introduced population of longtail macaques on Mauritius have been extensively used in recent research. This population has low MHC gene diversity, and is thus regarded as a valuable resource for research.

Methods We investigated the genetic diversity of this population using multiple molecular markers located in mitochondrial DNA and microsatellite DNA loci on the autosomes and the Y chromosome. We tested samples from 82 individuals taken from seven study sites.

Results and conclusions We found this population to be panmictic, with a low degree of genetic variability. On the basis of an mtDNA phylogeny, we inferred that these macaques' ancestors originated from Java in Asia. Weak gametic disequilibrium was observed, suggesting decay of non-random associations between genomic genes at the time of founding. The results suggest that macaques bred in Mauritius are valuable as model animals for biomedical research because of their genetic homogeneity.

Introduction

At present, longtail macaques (*Macaca fascicularis*) bred in and exported from Mauritius are frequently used in biomedical research [1]. This African colony of Asian macaques is the result of human introduction, probably during the Dutch occupation, or even the preceding Portuguese occupation of the island in the late 16th or early 17th century [26, 27]. There were estimated to be 25,000–35,000 of these macaques present in Mauritius in the 1980s [27]. The unique features of individuals of this population have been reported in previous studies, in which lower rates of pathogenic and viral infections [15] and larger body mass were found relative to conspecific macaques in their native Asian habitats [4].

The results of biomedical experiments involving monkey models, for example, studies of transplantation and cellular immunity, can vary depending on the origin of the monkeys [14, 16]. For longtail macaques, great diversity in major histocompatibility complex (MHC) genes and their differentiation among source countries have been reported [12, 31, 32]. The longtail macaques on Mauritius have a low degree of variation in MHC genes [2, 12, 14], and they are thus regarded as a valuable resource for biomedical research [12]. To better characterize the Mauritian macaque population, we studied their genetic diversity via study of polymorphisms in autosomal and Y-chromosomal microsatellite DNAs, and mitochondrial DNA. We aimed to determine (1) whether the population on the island of Mauritius is panmictic or structured; (2) whether the

population retains any sign of founding from Asia; (3) where the ancestors of this population originated from. DNA samples were prepared from frozen bloods that had been collected 16 years ago from individuals at various sites on the island. The degree of genetic diversity, population structure and origin of the population founders were assessed using genetic markers.

Materials and methods

Samples and DNA markers

The samples examined in this study were collected in 1989, and blood protein polymorphism data for these samples have been reported previously [11]. We examined 82 samples taken from individuals at seven different sites on the island (Fig. 1, Table 1). Habitats of macaques were not distributed uniformly in Mauritius, particularly scarce in the northern part of the island [27]. We tried to sample representative sites to test the genetic structure of the island population. The sampling was conducted during a capturing season of feral individuals for breeding. Although the sample size is small, we believe that samples in this study provide valuable information on genetic features of exported macaques for biomedical research. DNA was extracted from blood cells that had been stored at -80°C since the time at which the protein analysis was carried out. DNA samples were prepared from $200\ \mu\text{l}$ of the frozen blood cells by using a QuickGene-800 automatic nucleic acid isolation system (Fuji Photo Film Co., Ltd, Life Science Products Division, Tokyo, Japan). The yield of total genomic DNA using this system was $10\text{--}120\ \text{ng}/\mu\text{l}$.

Three kinds of genetic markers were used in this study: (1) autosomal microsatellite DNA markers (10 loci); (2) Y-chromosomal microsatellite DNA markers (three linked loci in the non-recombinational region); and (3) mitochondrial DNA (mtDNA) markers. All of the autosomal microsatellite loci contained tetranucleotide repeats. The combination of alleles from each of the three Y microsatellite loci defined a specific Y haplotype. Sequencing was performed for mtDNA analysis.

Microsatellite DNA typing

For microsatellite DNA typing, we examined a total of 10 homologues of short tandem repeat loci known in the human genome: *D1S533* (rhesus chromosome 1, location unknown), *D1S548* [rhesus chromosome 1, location 848.8 cR (centiRay in radiation hybrid mapping)], *D5S1470* (rhesus chromosome 6, location 70.5–106.2 cR), *D6S493* (rhesus chromosome 4, loca-

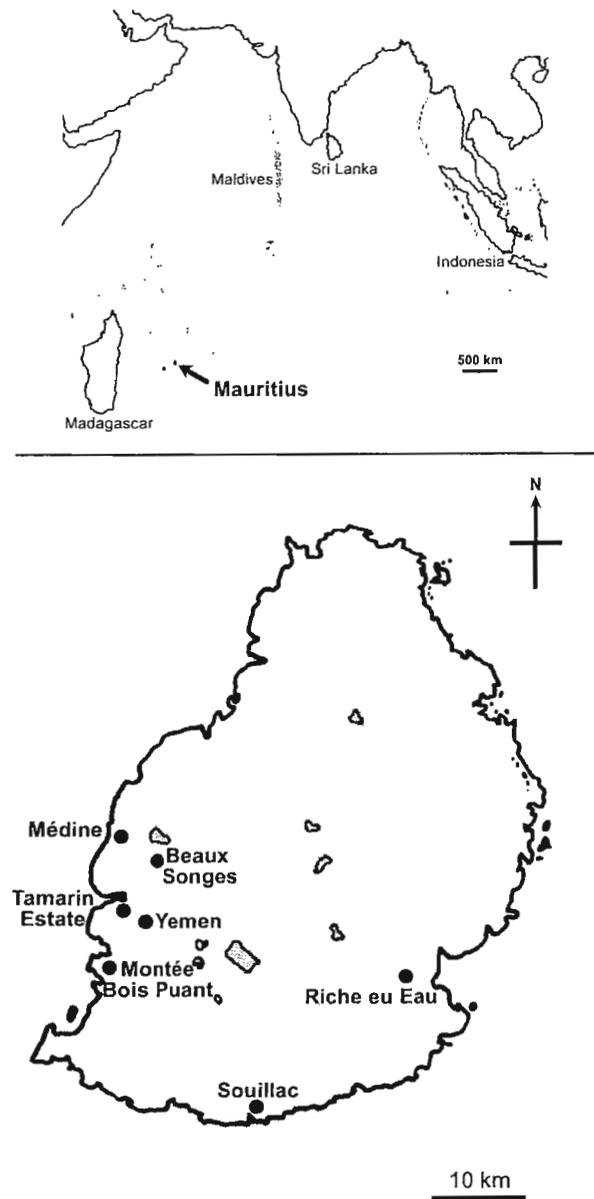


Fig. 1 Map showing the geographical location of Mauritius and the sampling sites on the island.

tion unknown), *D7S821* (rhesus chromosome 3, location 224.9 cR), *D10S611* (rhesus chromosome 9, location 30.2 cR), *D14S306* (rhesus chromosome 7, location 231.6 cR), *D17S1290* (rhesus chromosome 16, location 167.8 cR), *D19S582* (rhesus chromosome 19, location unknown) and *D20S484* (rhesus chromosome 10, location unknown) [17, 23]. Those microsatellite loci were used in this study because they had been known polymorphic in Japanese macaques (*M. fuscata*) (T. Shotake and A. Yamane, personal communication). We performed multiplex PCRs using

Table 1 Genetic diversity of autosomal microsatellite loci and results of a test for random mating in the whole population

Locus	N ¹	k ²	H _o ³	H _e ⁴	F _{is} ⁵	p ⁶ -value
<i>D1S533</i>	82	7	0.707	0.792	+0.107	0.144
<i>D1S548</i>	80	4	0.563	0.533	-0.055	0.035*
<i>D5S1470</i>	82	4	0.341	0.336	-0.018	1
<i>D6S493</i>	80	4	0.450	0.476	+0.054	0.241
<i>D7S821</i>	81	7	0.914	0.826	-0.107	0.659
<i>D10S611</i>	82	8	0.683	0.670	-0.020	0.924
<i>D14S306</i>	82	10	0.793	0.752	-0.055	0.109
<i>D17S1290</i>	82	10	0.720	0.799	+0.100	0.024*
<i>D19S582</i>	80	5	0.688	0.742	+0.047	0.387
<i>D20S484</i>	82	4	0.756	0.705	-0.072	0.491
(mean)		6.3	0.662	0.663		
all loci		$\chi^2 = 29.65$	df = 20	$P = 0.076$		

¹Number of samples.²Number of alleles.³Observed proportion of heterozygosity for the whole population.⁴Expected proportion of heterozygosity for the whole population.⁵Population inbreeding measure.⁶Fisher's exact probability test; * $P < 0.05$.

corresponding primer pairs for human loci under thermal cycling conditions of 5 min at 95°C; then 35 cycles of 30 s at 95°C, 30 s at 54°C, and 30 s at 72°C; followed by a final extension of 5 min at 72°C. A 12.5 μ l of PCR reaction mixture contained 10–30 ng genomic DNA, 1 \times buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂⁺], 1.875 μ M each primer, 150 μ M each dNTP, 0.625 U *TaKaRa Taq* polymerase. For the Y chromosome, we examined three loci: *DYS472*, *DYS569* and *DYS645* [5]. Multiplex genotyping was performed by using touchdown PCR with eight cycles of 60 s at 94°C, 60 s at 51°C (-0.5 per cycle), and 60 s at 60°C; then 32 cycles of 60 s at 94°C, 60 s at 47°C, 60 s at 60°C; and a final extension of 10 min at 60°C.

The DNA fragment analyses were carried out by applying the obtained PCR products diluted 20–30 times with water to an Abi Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif.) together with a molecular size standard (GeneScan 400HD ROX Size Standard; Applied Biosystems). The output files were analyzed using Genotyper software (Applied Biosystems) to determine genotypes.

DNA sequencing

Partial sequences of the mtDNA non-coding region (570–571 bp), encompassing the second hypervariable sequence (HVSII) region, were analyzed following the methods described in a previous study [7]. The DNA sequence corresponds to the nucleotides 42–605 in a registered complete mtDNA sequence of the rhesus macaque (accession no. NC_005943) [6]. We sequenced this mtDNA region because we had seen its great variations in Japanese macaques [10]. DNA sequences

were verified with Sequence Navigator (Applied Biosystems) to confirm the correspondence of the forward and reverse sequences. Multiple alignments for sequence comparisons were produced using ClustalX (1.8) software [29] and Genetyx-Mac Version 13 (Genetyx Corporation, Tokyo, Japan) using default settings. The DNA sequences were deposited in the DDBJ/EMBL/GenBank databases under the accession nos. AB281359–AB281367.

Data analysis

The genetic structure of the Mauritian macaque population was analyzed by using autosomal microsatellite DNA genotype data. Random mating was tested for both the population of the whole island and each study site with a Fisher's exact probability test implemented in Genepop software [22]. Here, the null hypothesis of random union of gametes was rejected when the sum of the probabilities for allele distribution under the limit of total allele counts exceeded a significant level. Nucleotide diversity [18] was calculated for each study site by using Arlequin version 2.0 software [25] and analysis of molecular variance (AMOVA) was performed to evaluate population subdivision in Mauritius, also by using Arlequin.

Genotypic disequilibrium for each locus pair across the whole island population was tested with a Fisher's exact probability test in Genepop. Here, the null hypothesis of independence of genotypes at compared loci was rejected when the sum of the probabilities for genotype distribution under the limit of total counts exceeded a significant level. Deviation from expected heterozygosity at mutation-drift equilibrium was tested

to determine whether a population bottleneck had occurred during the founding event. In the early stage of population bottleneck, alleles are lost in general faster than heterozygosity. This difference of decay speed allows testing the temporal excess of heterozygotes with respect to the observed number of alleles [3]. Such condition is expected to continue only for a short time after the event of size decrease. That test assumed three mutation models, the infinite allele model (IAM), the two-phased model (TPM) and the stepwise mutation model (SMM), as implemented in Bottleneck software [3].

The molecular phylogenies of mtDNA haplotypes were analyzed by distance, maximum likelihood (ML) and Bayesian Markov chain Monte Carlo (MCMC) methods. Seven reference sequences were compared with those of Mauritius macaques: Java (Jatibarang, accession number AB281359), Sumatra (Tabuan, accession number AB281360), Borneo (Pangkalanbun, accession number AB281361), Bali (Ubud, accession number AB281362), Mindanao (Cotabato, accession number AB281363), Cambodia (Ban Ta Poy, accession number AB281364) and China (imported into Japan, probably originating in Vietnam, accession number AB281365). The distance method used neighbor joining (NJ) clustering [24] with uncorrected *p*-distance. The validity of branching was tested by bootstrap support with 1000 replications using PAUP* version 4.0b10 [28]. To determine a suitable substitution model in the ML analysis, the sequence data set was analyzed using Modeltest 3.6 [21]. The result of a hierarchical likelihood ratio test showed that the GTR + I + G model was the best fit model of base substitution. Using this model, ML analysis was performed in PAUP* using the heuristic search option with tree-bisection-reconnection (TBR) branch swapping. Branch support was estimated by bootstrapping with 1000 replications. The Bayesian analysis was performed in MrBayes 3.0b4 [8] and the posterior probability distribution of trees was approximated using samples taken every 100 steps over 1,000,000 MCMC cycles, after discarding a burn-in of 2000 cycles (20% of total generations).

Results

DNA polymorphism

All autosomal microsatellite loci investigated in this study were polymorphic, with 4–10 alleles (Table 1). The estimates of diversity measured using the expected proportion of heterozygosity for the whole island population ranged from 33.6% at the *D5S1470* locus to 82.6% at the *D7S821* locus. The estimated frequencies of alleles at each study site are given in the Appendix.

There were two types of mtDNA in the 74 samples from individuals at the seven study sites (Table 2). Here, eight samples could not be tested due to failure in PCR amplification with unknown reason. An insertion mutation in a stretch of C repeats was involved in the polymorphism. The shorter haplotype had seven C repeats in the sequence and the longer had eight repeats. Except for this mutation, all other nucleotides were identical between the two mtDNA haplotypes. Study of the geographical distribution of haplotypes indicated that the two haplotypes were not distributed uniformly; most of the study sites were characterized by having the shorter haplotype, and only two sites contained individuals with the longer haplotype (Table 2). When male samples were removed, all of the study sites were characterized by having a single mtDNA haplotype, and only a single site (Souillac) had the longer haplotype.

No size polymorphism was detected at any of the three Y chromosomal microsatellite loci in the 38 males examined, so the composite haplotype defined by combining the three loci was monomorphic in the Mauritian population.

Properties of the population

The results of the test for random mating are given in Table 1. Although the locus by locus test indicated two cases of significant deviation from random mating, the overall result from the 10 loci suggested panmictic conditions in the Mauritian population. Similarly, the results of tests at each study site were all statistically non-significant (Table 2). The estimated gene diversity in terms of the expected proportion of heterozygosity is given in Table 2. The estimates significantly depended on sample size ($r = +0.76$; $t = 2.61$, $P < 0.05$). These results suggest that there are few indications of population subdivision and that the macaque population can be regarded as a whole as a single breeding unit. AMOVA also suggested a low degree of differentiation among study sites: 97% of the total molecular variation was attributed to that occurring within sub-populations (sites).

The results of pairwise comparisons of 10 autosomal microsatellite loci to assess genotypic disequilibrium are summarized in Table 3. Of 45 possible combinations, four (8.9%) including the linked loci in the rhesus genome (*DIS533* and *DIS548*) were significant for non-random association of alleles.

The results of tests for population bottleneck varied among marker loci, and the overall evaluation by the Wilcoxon test detected significant heterozygote excess in IAM but not in TPM and SMM (Table 4). As the

Table 2 Results of Fisher's exact probability test for random mating, gene diversity and observed mtDNA types for each subpopulation (study site)

Study site (abbreviation)	n ¹	random mating test ²			Gene diversity Mean ± SD	mtDNA type ³		
		χ^2	df	P-value		S	L	NT
Beaux Songes (BS)	15	17.31	20	0.63	0.700 ± 0.388	15	0	0
Médine (MD)	6	8.01	18	0.98	0.618 ± 0.356	5	0	1
Montée Bois Puant (MP)	7	3.23	18	1	0.581 ± 0.332	7	0	0
Rihe eu Eaus (RE)	16	12.19	20	0.91	0.680 ± 0.380	12	2 ⁴	2
Souillac (SL)	6	7.95	20	0.99	0.636 ± 0.365	1 ⁴	5	0
Tamarin Estate (TE)	16	17.71	20	0.61	0.656 ± 0.358	11	0	5
Yemen (YM)	16	18.95	20	0.53	0.650 ± 0.351	16	0	0
Total	82					67	7	8

¹Number of samples.

²Fisher's exact probability test.

³S = mtDNA C type with seven C repeats; L, mtDNA type with eight C repeat; NT, not tested.

⁴Detected only in male samples.

IAM model seems to be less plausible for microsatellite DNA, it was concluded that the sign of heterozygote excess expectable in the initial stage of population bottleneck was weak in the Mauritian population.

Origin of the population

The origin of the ancestors of the Mauritian macaques was inferred by comparing the obtained mtDNA sequences with those of macaques living in Asian habitats. The haplotypes found in Mauritius were clustered together with those in the Asian region including Sumatra, Java, Bali, Borneo and Mindanao (Fig. 2). The Mauritian haplotypes showed proximity with high bootstrap values or posterior probability to the Java haplotype and were unquestionably distant from haplotypes in the Indo-China region.

Discussion

Our genetic study revealed evidence of low levels of geographical differentiation and genetic homogeneity of the macaque population on Mauritius. This genetic homogeneity is typically reflected in a low diversity in mtDNA and Y chromosomal variations. MtDNA result agrees to those in a restriction enzyme analysis of non-coding region (ca. 1.8 kb) for 19 samples in which two closely related haplotypes were detected [10], and in a sequencing analysis of coding region (1.5 kb) for 10 samples in which all samples carried identical sequence [30]. Lack of YDNA polymorphism was also reported in a sequencing analysis of TSPY/SRY genes for 10 samples [30]. These features suggest small founder size during colonization of the area with individuals from Asia or loss of genetic variation

through a bottleneck after the founding event. In contrast, no autosomal microsatellite DNA loci tested here was fixed to a single allele, indicating that the founder size was not small enough to cause homogeneity in nuclear DNAs. Alternatively, most of the alleles could be unique mutants that occurred after introduction. The latter idea could be tested by comparing the observed allele types with those in countries where the macaque is native, but no data is available to us at present.

The level of genetic variability was larger in autosomal microsatellite loci than mtDNA and YDNA. Unfortunately, we do not have comparable microsatellite data of local populations for the same species to evaluate the level of variability in Mauritius. Comparing other macaque species, the mean of expected heterozygosity of Mauritian macaques for 10 microsatellite loci (0.663 in Table 1) was smaller than those in local populations of Japanese macaques for the same set of loci, usually >0.7 in the mainland population (unpublished data). Although there may be a difference between different species in the degrees of variability among marker loci, this comparison also suggests low genetic variability of nuclear genes in the Mauritian macaque.

The present study generally supported the conclusion of previous studies with respect to the origin and population structure of Mauritian macaques. Our result agreed with the conclusion of Java origin in a protein study in which Java population share full set of allele types with Mauritius population [11]. Restriction fragment length polymorphism in mtDNA also suggested their proximity to Indonesian macaques, although island origin was not specified [13]. Recently, a comparative study of mtDNA sequences based on

Table 3 Fisher's exact probability test for genotypic disequilibrium for each locus pair across the whole population. Alleles were selectively combined to perform the statistical test

Locus pair	χ^2	df	P-value
¹ D1S533 & D1S548	8.877	2	0.012*
D1S533 & D5S1470	0.359	2	0.836
D1S533 & D6S493	1.012	2	0.603
D1S533 & D7S821	3.798	2	0.150
D1S533 & D10S611	2.505	2	0.286
D1S533 & D14S306	5.042	2	0.080
D1S533 & D17S1290	1.073	2	0.585
D1S533 & D19S582	1.810	2	0.405
D1S533 & D20S484	0.810	2	0.667
D1S548 & D5S1470	1.071	2	0.585
D1S548 & D6S493	0.597	2	0.742
D1S548 & D7S821	11.365	2	0.003**
D1S548 & D10S611	0.241	2	0.886
D1S548 & D14S306	2.115	2	0.347
D1S548 & D17S1290	0.778	2	0.678
D1S548 & D19S582	1.745	2	0.418
D1S548 & D20S484	4.726	2	0.094
D5S1470 & D6S493	2.132	2	0.344
D5S1470 & D7S821	5.121	2	0.077
D5S1470 & D10S611	0.901	2	0.637
D5S1470 & D14S306	7.994	2	0.018*
D5S1470 & D17S1290	1.585	2	0.453
D5S1470 & D19S582	0.132	2	0.936
D5S1470 & D20S484	1.909	2	0.385
D6S493 & D7S821	0.854	2	0.652
D6S493 & D10S611	2.028	2	0.363
D6S493 & D14S306	1.934	2	0.380
D6S493 & D17S1290	1.677	2	0.432
D6S493 & D19S582	3.438	2	0.179
D6S493 & D20S484	4.731	2	0.094
D7S821 & D17S1290	3.377	2	0.185
D7S821 & D14S306	0.614	2	0.736
D7S821 & D10S611	1.653	2	0.438
D7S821 & D19S582	0.580	2	0.748
D7S821 & D20S484	0.143	2	0.931
D10S611 & D14S306	0.100	2	0.951
D10S611 & D17S1290	3.936	2	0.140
D10S611 & D19S582	0.522	2	0.770
D10S611 & D20S484	1.318	2	0.517
D14S306 & D17S1290	4.461	2	0.107
D14S306 & D19S582	2.984	2	0.225
D14S306 & D20S484	1.125	2	0.570
D17S1290 & D20S484	10.563	2	0.005**
D17S1290 & D19S582	1.612	2	0.447
D19S582 & D20S484	4.360	2	0.113

¹Linked loci in rhesus genome.* $P < 0.05$; ** $P < 0.01$.

the coding region spanning the 12S ribosomal gene, tRNA-val and the 16S ribosomal gene found a proximity of Mauritian type to Sumatra types with a possibility of different paternal origin from peninsula Malaysia inferred from Y chromosomal DNA [30]. As

mtDNA diversity tends to be large in the Javanese and its surrounding populations [20], further investigation of geographical distribution of mtDNA haplotypes is necessary to better pinpoint the origin of the Mauritian macaques. Clarification of microsatellite DNA variations in native countries in future studies may help to evaluate the origin and to test the possibility of different maternal and paternal ancestry.

The tendency of weak population subdivision in Mauritius was suggested from the previous protein study in which 9% of the total molecular variation was attributed to that occurring between subpopulations (sites) [11]. This is larger than the estimate (3%) from microsatellite DNA in this study. The difference in the degree of population subdivision may result from bias in marker locus sampling or individual sampling in allele frequency estimation. Consequently, the level of nuclear differentiation in Mauritius is low comparing the protein estimates for longtail macaques in Java (30%), Sumatra (18%) and Bali (10%) [9]. Although the diversity of vegetation in Mauritius seems to cause differences in macaque group density [27], variation in the vegetation does not affect genetic differentiation among groups on the island. Intergroup transfer of adult males may prevent the subdivision of populations.

There was no strong evidence of gametic disequilibrium indicating a remain of sign of bottleneck in the Mauritian population. Such disequilibrium caused by a population bottleneck usually decays over generations. Under random mating conditions as seen in Mauritius, the decay can proceed quickly for unlinked loci and reduce the initial disequilibrium state to levels of 3.1% and 0.1% after five and 10 generations (equivalent to approximately 50 and 100 years for macaques [19]), respectively. Notably, one significant case of disequilibrium was found in microsatellite DNA loci on the same chromosome (*D1S533* and *D1S548*), rhesus chromosome 1 [23] (Table 3). Although the genes of this genome seem to have been shuffled substantially, the non-random association of tightly linked genes could be maintained in the Mauritian macaques due to the time that has elapsed since the founding event. This genetic feature may be of value for use of these macaques in laboratory studies.

Export of Mauritian macaques relies on the supply of breeding males and females from free-ranging feral groups. The results of our study indicate that the genetic quality of bred macaques could be maintained regardless of the sampling sites used to collect individuals for breeding. Because of their characteristic low genetic diversity and low levels of pathogens, Mauri-

Table 4 Test for a population bottleneck for each locus using different mutation models

Locus	Observed			IAM				TPM				SMM			
	n	k _o	H _e	H _{eq}	SD	DH/SD	P-value	H _{eq}	SD	DH/SD	P-value	H _{eq}	SD	DH/SD	P-value
D1S533	164	7	0.792	0.589	0.143	1.417	0.0160*	0.688	0.092	1.132	0.0630	0.757	0.058	0.593	0.3000
D1S548	160	4	0.533	0.396	0.185	0.742	0.2870	0.482	0.157	0.329	0.4440	0.578	0.112	-0.399	0.2620
D5S1470	164	4	0.336	0.401	0.181	-0.362	0.3530	0.490	0.154	-1.001	0.1720	0.582	0.105	-2.353	0.0360*
D6S493	160	4	0.476	0.395	0.182	0.445	0.4020	0.486	0.155	-0.068	0.3790	0.574	0.111	-0.889	0.1640
D7S821	162	7	0.826	0.600	0.142	1.592	0.0060**	0.683	0.095	1.507	0.0100*	0.760	0.054	1.208	0.0610
D10S611	164	8	0.670	0.629	0.134	0.303	0.4750	0.716	0.086	-0.542	0.2460	0.792	0.046	-2.640	0.0220*
D14S306	164	10	0.752	0.695	0.115	0.496	0.3720	0.778	0.063	-0.425	0.2820	0.835	0.035	-2.368	0.0330*
D17S1290	164	10	0.799	0.695	0.113	0.924	0.1460	0.780	0.062	0.305	0.4500	0.837	0.034	-1.114	0.1310
D19S582	160	5	0.742	0.478	0.177	1.493	0.0190*	0.568	0.134	1.306	0.0390*	0.657	0.091	0.933	0.1540
D20S484	164	4	0.705	0.392	0.182	1.724	0.0080**	0.487	0.154	1.425	0.0240*	0.575	0.108	1.206	0.0550
Overall (Wilcoxon test: one tail for heterozygote excess)															
				IAM: P = 0.0015**				TPM: P = 0.1377				SMM: P = 0.8125			

IAM, infinite allele model; TPM, two-phased model; SMM, stepwise mutation model.

k_o, observed number of alleles; H_e, expected heterozygosity; H_{eq}, heterozygosity at mutation-drift equilibrium; SD, standard deviation. DH = H_e - H_{eq}; *P < 0.05; **P < 0.01.

NJ / ML / Bayes

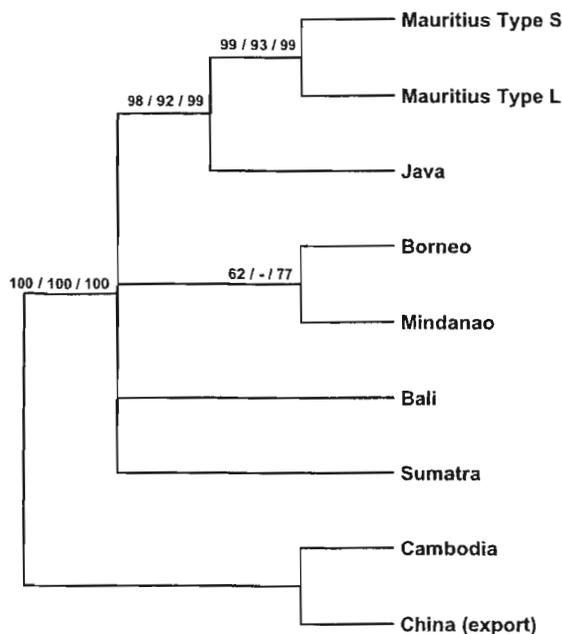


Fig. 2 Molecular phylogenetic tree based on mtDNA sequences. This tree is a 50% majority-rule consensus tree showing percentage bootstrap values of NJ and ML clustering and percentage Bayesian posterior probabilities (Bayes).

tian macaques can be considered to be suitable experimental animals because the genetic homogeneity of individuals supplied from the breeding facilities can improve the general reproducibility of biomedical experiments.

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Appendix

Estimated frequencies of microsatellite alleles in longtail macaque populations on Mauritius. Numbers in parenthesis indicate sample sizes. For abbreviations of study sites, see Table 2

Locus	Allele	Total (82)	Study site						
			TE (16)	RE (16)	SL (6)	YM (16)	BS (15)	MP (7)	MD (6)
<i>D19S582</i>	151	0.0250	0.0000	0.0667	0.0000	0.0312	0.0000	0.0714	0.0000
	155	0.2125	0.0000	0.3000	0.2500	0.2188	0.1429	0.5000	0.3333
	163	0.1437	0.1562	0.1333	0.1667	0.1875	0.0000	0.1429	0.3333
	167	0.3562	0.4688	0.2667	0.5000	0.1562	0.6429	0.1429	0.2500
	171	0.2625	0.3750	0.2333	0.0833	0.4062	0.2143	0.1429	0.0833
<i>D1S548</i>	195	0.1750	0.1250	0.2000	0.1667	0.3125	0.1429	0.0714	0.0833
	199	0.1813	0.3125	0.1667	0.0833	0.1875	0.1786	0.0714	0.0833
	203	0.6375	0.5625	0.6333	0.7500	0.5000	0.6786	0.8571	0.7500
<i>D6S493</i>	207	0.0063	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0833
	251	0.6875	0.6562	0.6667	0.8333	0.8125	0.5357	0.6429	0.7500
	259	0.0125	0.0625	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	267	0.2188	0.0625	0.2333	0.1667	0.1562	0.4286	0.2857	0.2500
<i>D7S821</i>	271	0.0812	0.2188	0.1000	0.0000	0.0312	0.0357	0.0714	0.0000
	261	0.2222	0.2333	0.2500	0.3333	0.1875	0.1000	0.2857	0.3333
	265	0.1605	0.0333	0.1875	0.1667	0.2188	0.1000	0.2857	0.2500
	281	0.1296	0.1333	0.0938	0.0000	0.2500	0.1333	0.0000	0.1667
	286	0.0062	0.0333	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	291	0.0802	0.1333	0.1250	0.0000	0.0938	0.0667	0.2857	0.0000
<i>D14S306</i>	295	0.1852	0.1333	0.1875	0.2500	0.0938	0.2667	0.1429	0.1667
	299	0.2160	0.3000	0.1562	0.2500	0.1562	0.3333	0.0000	0.0833
	164	0.0061	0.0000	0.0312	0.0000	0.0000	0.0000	0.0000	0.0000
	168	0.1829	0.0312	0.3125	0.2500	0.1250	0.2000	0.2857	0.1667
	172	0.4329	0.4062	0.4062	0.3333	0.5000	0.3333	0.5714	0.5833
	177	0.0366	0.1562	0.0000	0.0000	0.0000	0.0333	0.0000	0.0000
	181	0.0976	0.1250	0.0938	0.0833	0.0625	0.1333	0.0000	0.1667
	185	0.0183	0.0625	0.0000	0.0000	0.0312	0.0000	0.0000	0.0000
	188	0.1098	0.0938	0.0312	0.1667	0.1875	0.2000	0.0000	0.0000
	192	0.0183	0.0312	0.0000	0.0000	0.0312	0.0000	0.0000	0.0833
<i>D10S611</i>	196	0.0915	0.0938	0.1250	0.1667	0.0312	0.1000	0.1429	0.0000
	204	0.0061	0.0000	0.0000	0.0000	0.0312	0.0000	0.0000	0.0000
	162	0.0305	0.0000	0.0625	0.0000	0.0312	0.0000	0.0000	0.1667
	172	0.0122	0.0312	0.0312	0.0000	0.0000	0.0000	0.0000	0.0000
	187	0.0061	0.0000	0.0000	0.0000	0.0000	0.0333	0.0000	0.0000
	191	0.3049	0.4688	0.2812	0.0833	0.3125	0.2667	0.2143	0.3333
	195	0.4573	0.3750	0.5000	0.5833	0.4375	0.5000	0.4286	0.4167
<i>D5S1470</i>	200	0.0061	0.0000	0.0000	0.0833	0.0000	0.0000	0.0000	0.0000
	204	0.1768	0.1250	0.1250	0.2500	0.1875	0.2000	0.3571	0.0833
	212	0.0061	0.0000	0.0000	0.0000	0.0312	0.0000	0.0000	0.0000
	196	0.0061	0.0000	0.0000	0.0000	0.0000	0.0333	0.0000	0.0000
	200	0.7927	0.7188	0.8125	0.6667	0.8438	0.6667	1.0000	1.0000
	204	0.1951	0.2812	0.1875	0.3333	0.1250	0.3000	0.0000	0.0000
	208	0.0061	0.0000	0.0000	0.0000	0.0312	0.0000	0.0000	0.0000

Appendix (Continued)

Locus	Allele	Total (82)	Study site						
			TE (16)	RE (16)	SL (6)	YM (16)	BS (15)	MP (7)	MD (6)
<i>D17S1290</i>	238	0.0061	0.0000	0.0000	0.0000	0.0312	0.0000	0.0000	0.0000
	242	0.2439	0.1250	0.1250	0.1667	0.3750	0.4000	0.2143	0.2500
	246	0.2805	0.4688	0.4062	0.3333	0.0938	0.1667	0.2857	0.1667
	250	0.1341	0.0625	0.2188	0.0833	0.0625	0.1667	0.1429	0.2500
	254	0.0366	0.0938	0.0312	0.0000	0.0625	0.0000	0.0000	0.0000
	258	0.0305	0.0000	0.0312	0.0000	0.0312	0.0667	0.0000	0.0833
	262	0.0122	0.0000	0.0625	0.0000	0.0000	0.0000	0.0000	0.0000
	266	0.0366	0.0625	0.0000	0.0000	0.0938	0.0000	0.0000	0.0833
	270	0.2134	0.1875	0.1250	0.3333	0.2500	0.2000	0.3571	0.1667
	274	0.0061	0.0000	0.0000	0.0833	0.0000	0.0000	0.0000	0.0000
<i>D20S484</i>	120	0.1220	0.1562	0.0938	0.0000	0.2500	0.1333	0.0000	0.0000
	124	0.4146	0.3750	0.4062	0.5000	0.3125	0.5333	0.3571	0.5000
	128	0.2805	0.2188	0.3750	0.2500	0.2188	0.3000	0.4286	0.1667
	144	0.1829	0.2500	0.1250	0.2500	0.2188	0.0333	0.2143	0.3333
<i>D1S533</i>	186	0.3110	0.1250	0.4375	0.1667	0.4688	0.2333	0.2857	0.4167
	198	0.0915	0.1875	0.0625	0.5000	0.0000	0.0333	0.0000	0.0000
	210	0.0244	0.0000	0.0000	0.0000	0.0312	0.1000	0.0000	0.0000
	214	0.2256	0.3438	0.2188	0.3333	0.2812	0.0667	0.1429	0.1667
	218	0.1951	0.1875	0.0625	0.0000	0.0312	0.4667	0.5000	0.1667
	226	0.1341	0.0938	0.2188	0.0000	0.1562	0.1000	0.0714	0.2500
	230	0.0183	0.0625	0.0000	0.0000	0.0312	0.0000	0.0000	0.0000

Unusually High Frequency MHC Class I Alleles in Mauritian Origin *Cynomolgus* Macaques

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Acute shortages of Indian origin Rhesus macaques significantly hinder HIV/AIDS research. Cellular immune responses are particularly difficult to study because only a subset of animals possess MHC class I (MHC I) alleles with defined peptide-binding specificities. To expand the pool of nonhuman primates suitable for studies of cellular immunity, we defined 66 MHC I alleles in *Cynomolgus* macaques (*Macaca fascicularis*) of Chinese, Vietnamese, and Mauritian origin. Most MHC I alleles were found only in animals from a single geographic origin, suggesting that *Cynomolgus* macaques from different origins are not interchangeable in studies of cellular immunity. Animals from Mauritius may be particularly valuable because >50% of these *Cynomolgus* macaques share the MHC class I allele combination *Mafa-B*430101*, *Mafa-B*440101*, and *Mafa-B*460101*. The increased MHC I allele sharing of Mauritian origin *Cynomolgus* macaques may dramatically reduce the overall number of animals needed to study cellular immune responses in nonhuman primates while simultaneously reducing the confounding effects of genetic heterogeneity in HIV/AIDS research. *The Journal of Immunology*, 2005, 175: 5230–5239.

Nonhuman primates are widely used to model pathogenic human diseases. For pathogens such as HIV/AIDS where no representative small animal model is available, nonhuman primates are indispensable for pathogenesis studies and preclinical vaccine evaluation (1, 2). Rhesus macaques (*Macaca mulatta*) currently dominate nonhuman primate AIDS research; of the 287 vaccine trials indexed in the Nonhuman Primate HIV/SIV Vaccine Database, >200 have used this species.

The supply of Indian Rhesus macaques, however, is dwindling (3). Since 1978, India has banned the export of feral Rhesus macaques (4), making breeding programs the sole source of research animals. The supply of macaques produced by breeding has not kept pace with the demand (5). Additionally, the demand for Indian Rhesus macaques by biodefense and transplant researchers (6) is constraining the availability of animals for HIV research.

HIV vaccine research is disproportionately affected by the Indian Rhesus macaque shortage. Vaccine studies are inherently expensive because populations large enough to detect statistically meaningful differences between vaccinated macaques and vaccine naive controls must be included. For trials studying CD8⁺ T lymphocyte responses, both groups of animals are often MHC class I (MHC I)² allele matched to quantitate immune responses against the same epitopes (7, 8). This has exacerbated the shortage and demand for MHC-defined Indian Rhesus macaques (5) because "high frequency" alleles such as Mamu-A*01 are only present in 20–25% of macaques (9). Moreover, postacute viral loads are in-

creasingly being used as vaccine trial endpoints (10–13). These studies are particularly susceptible to differences in MHC I genotypes, as certain alleles, such as Mamu-A*01 (14, 15), Mamu-B*17 (16), and Mamu-A*1303 (17) predispose animals to favorable SIV outcomes.

Fortunately, Indian Rhesus macaques are not unique in their susceptibility to pathogenic SIV infection. Chinese Rhesus macaques can also be infected with SIV (18, 19), though these animals do not possess MHC-I α alleles, such as Mamu-A*01, that are common in Indian Rhesus macaques (20). *Cynomolgus* macaques (*Macaca fascicularis*) represent another potential nonhuman primate model for SIV. More than 9,000 *Cynomolgus* macaques are imported for research each year (T. Demarcus, unpublished observations). *Cynomolgus* macaques, like Rhesus macaques, are geographically distributed throughout Asia (21). The most widely imported Asian *Cynomolgus* macaques originate in Vietnam, China, Indonesia, and the Philippines. Though the natural range of *Cynomolgus* macaques extends throughout Asia, the largest exporter is the Indian Ocean island of Mauritius. Between 5500 and 8000 feral and captive bred *Cynomolgus* macaques are exported from Mauritius each year (22).

Very little is known about MHC I genes of *Cynomolgus* macaques. In the 1980s, >30 MHC I serotypes were described (23), but these serotypes were never resolved at the molecular level. One-dimensional isoelectric focusing (1D-IEF) of MHC I proteins immunoprecipitated from *Cynomolgus* macaques suggests that these animals express between five and seven MHC I proteins (24). Recently, 14 MHC I A locus allele sequences (25) and 26 MHC I B locus alleles (26) were described. Additionally, there is strong evidence that the B locus can be duplicated, as has been previously observed in Rhesus macaques (27).

Others have observed that *Cynomolgus* macaque origin can influence the success of kidney allografts (28) and susceptibility to *Plasmodium coatneyi* infections (29). We hypothesized that these outcome disparities are due, at least in part, to differences in the regional distribution of MHC I allele repertoires. If proven correct, the origin of *Cynomolgus* macaques may be a critical and overlooked variable that prejudices the host response to pathogens

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² Abbreviations used in this paper: MHC I, MHC class I; 1D-IEF, one-dimensional isoelectric focusing; RSCA, reference-strand conformational analysis.

including SIV. To test this hypothesis, we identified >60 new MHC I alleles from *Cynomolgus* macaques of Vietnamese, Chinese, and Mauritian origin. We did not detect the previously described MHC I alleles in any of the animals examined, suggesting that these alleles were identified in animals from a different origin. Mauritian animals exhibited an unexpected degree of allele sharing, prompting the development of genotyping assays for five common alleles. More than 50% of Mauritian *Cynomolgus* macaques possessed a combination of three MHC I alleles, at least one of which is expressed as protein on the cell surface. We speculate that animals possessing this allele combination may be uniquely valuable for SIV and biodefense projects that require monitoring of CD8⁺ T lymphocyte responses or MHC I matching.

Materials and Methods

Animals and veterinary care

Vietnamese origin *Cynomolgus* macaques (Cy0051-Cy0090) were purchased from Covance Research Products. Chinese origin *Cynomolgus* macaques (Cy0091-Cy0110) were purchased from Central State Primates. The Vietnamese and Chinese *Cynomolgus* macaques were housed at the Wisconsin National Primate Research Center. All of these animals were cared for according to protocols approved by the University of Wisconsin Research Animal Resource Committee.

All experiments on Mauritian *Cynomolgus* macaques were performed on whole blood provided by Charles River Laboratories. Blood from eight monkeys (A1M–A8M) was initially obtained for MHC I allele discovery. Subsequently, blood from an additional 48 animals, obtained in three different shipments, was procured and used for MHC I allele genotyping.

MHC I allele cloning and sequencing

MHC I alleles were amplified by RT-PCR, cloned, and sequenced. The criteria that we used to define novel alleles by cloning and sequencing was intentionally stringent; an allele was defined by at least two full-length, nucleotide identical sequences. A sizable fraction of PCR-amplified MHC alleles contain sequence artifacts, including intramolecular recombinants and single nucleotide substitutions (30). To minimize these effects, the PCR conditions were carefully optimized, and high fidelity Phusion DNA polymerase was used in all amplifications. Nonetheless, a number of non-authentic sequences were obtained from each animal. For example, in animal A1M, 13 clones perfectly matched the Mafa-B*430101 sequence. An additional 16 clones were nonperfect matches to the Mafa-B*430101 sequence that were more closely related to this allele than to any other allele. Authentic MHC I alleles that are not as prominent in our transcript pool (31) may be overlooked with our stringent approach, however, we feel that this is preferable to describing "allele sequences" that may not be biologically relevant.

PBMC were collected from EDTA-treated whole blood by Ficoll-Paque (GE Health Sciences) density gradient purification. Total RNA was harvested from the PBMC with a Qiagen Total RNeasy kit (Qiagen) following the manufacturer's instructions. cDNA was synthesized with either SuperScript II or SuperScript III Reverse Transcriptase (Invitrogen Life Technologies). Oligo(dT)-primed reverse transcription was performed according to the manufacturer's protocol. The cDNA was amplified using PCR primers designed to maximize amplification of known Indian Rhesus macaque MHC I sequences. Each cDNA was amplified in two PCR, both of which used the same universal forward primer (5'*NotI*-MHC-full; 5'-GCGGCCGATGSSSGTCATGGCGCCSSG-3'). The 5'*NotI*-MHC-full oligonucleotide contains a *NotI* restriction site in addition to sequence from the first exon of MHC I sequences. Both reverse oligonucleotides, 3'*Alocus-KpnI* (5'-GGTACCTCACTTTACAAGCCGTGAGAGACAC-3') and 3'*Blocus-KpnI* (5'-GGTACCTCAAGCCGTGAGAGACWCATCAGACC-3'), contain *KpnI* restriction sites. These two primers differ in their MHC I exon 7 sequences, as MHC I A locus alleles possess nine additional nucleotides adjacent to the translational stop codon. Because the differences between these two primers are located centrally in the oligonucleotide, both reverse primers amplify MHC I A locus and B locus sequences. The PCR was performed using the DNA polymerase Phusion (Bio-Rad) and the following reaction conditions: 98°C for 30 s, 25 cycles of (98°C for 5 s, 65°C for 1 s, 72°C for 20 s), 72°C for 5 min, 4°C until time-of-use. Amplified cDNA was purified with either a QIAQUICK Gel Extraction kit or a QIAQUICK PCR Purification kit according to the manufacturer's protocol. The purified DNA was ligated into pCR-Blunt using the Invitrogen Zero Blunt cloning kit (Invitrogen Life Technologies). Between 48 and

192 clones/animal were minipreped and sequenced using the primers T7 (5'-TAATACGACTCACTATAGGG-3'), M13 (5'-CAGGAAACAGC TATGAC-3'), 5'RefStrand (5'-GCTACGTGGACGACACGC-3'), and 3'RefStrand (5'-CAGAAGGCACCACGACAGC-3') on an ABI 3730 DNA Analyzer (Applied Biosystems). Sequences were analyzed using software from Genecodes and Accelrys. Novel MHC I sequences were given GenBank accession nos. AY958087–AY958152.

Phylogenetic tree construction

Phylogenetic trees of the MHC I alleles were constructed by the neighbor-joining method (32) based on Kimura's two-parameter distance (33).

Generation of custom DNA size standards

A ROX-labeled size standard was prepared using amplicons based on the vector pcDNA3.1+ (Invitrogen Life Technologies). A universal ROX-labeled primer ROX-pcDNA3.1 + 2215-F (5'-ROX-AGACAATCGGCT GCTCTGAT-3') and a series of nested, unlabeled oligonucleotides were used to produce fragments of 110 (5'-ctcgtctcgcagrtcatca-3'), 203 (5'-caatagcagccagtccttc-3'), 310 (5'-gtagccggatcaagcgtatg-3'), 411 (5'-cct gatgctcttcgctcag-3'), 525 (5'-ggccatttccaccatgata-3'), and 621 (5'-cgc caagcttcagcaata-3') bp. Twenty-six larger size fragments, irrelevant to the MHC I genotyping, and ranging from 661 to 2324 bp, were also included in the size standard.

MHC I genotyping

Genotyping was performed by reference-strand conformational analysis (RSCA). Allele clones containing *Mafa-A*200101* (GenBank no. AY958088), *Mafa-B*370101* (GenBank no. AY958132), and *Mamu-B*07* (GenBank no. U41829) were PCR amplified using the labeled primer 6FAM-5'ShortRSCA (5'-[6FAM]AGGGGCCGAGTATTGGG-3') and the 5' phosphate-modified primer Short3'RSCA-P (5'-[Phos]TTCAGG GCGATGTAATCC-3'). Clones containing *Mafa-B*430101*, *-B*440101*, *-B*460101*, *-B*470101*, and *-B*510101* were PCR amplified using the 5' phosphate modified primer 5'ShortRSCA-P (5'-[Phos]AGGGGCCGAG TATTGGG-3') and the unlabeled primer Short3'RSCA (5'-TTCAGG GCGATGTAATCC-3'), generating a 217-bp amplicon. cDNA for genotyping was prepared as described above and was amplified with the 5'ShortRSCA-P and Short3'RSCA primers. All PCR were performed using the DNA polymerase Phusion and the reaction conditions described above. Following PCR, the antisense strands of the reference strand amplicons and the sense strands of the clone and cDNA amplicons were digested with λ exonuclease (Epicentre Technologies) according to the manufacturer's protocol. λ exonuclease selectively digests phosphorylated strands of dsDNA, reducing the formation of homoduplex and nonlabeled heteroduplex products in the heteroduplexing reaction. To form heteroduplexes, 1.5 μ l of exonuclease treated, FAM-labeled reference strand was mixed with either 1.5 μ l of exonuclease-treated product amplified from a DNA clone or 3 μ l of exonuclease-treated product amplified from *Cynomolgus* macaque cDNA. The heteroduplexing reactions were heated to 95°C for 4 min, cooled to 55°C for 5 min, and chilled to 15°C for 5 min. Eight microliters of the custom ROX size standard were added to each heteroduplex reaction, and these mixtures were purified over Sephadex-G50 columns and concentrated by vacuum. The concentrated products were resuspended in 2.0 μ l Dextran Blue/EDTA solution, and 1.5 μ l of the resuspension was applied to a 96 lane RapidLoad 2.0 membrane comb (Gel Company). The heteroduplexes were resolved on a 3% nondenaturing acrylamide gel run on an ABI 377 DNA Analyzer (Applied Biosystems). Electrophoresis was performed for 12 h at 1200 V, with the gel temperature set to 30°C.

After extracting data from the ABI 377 raw data files using Genescan 3.1 (Applied Biosystems), the Dax software package (Van Mierlo Software Consultancy) was used for all fragment analysis and sizing. Peak mobilities were converted from seconds to apparent base pairs by fitting the samples to a standard curve generated by the custom ROX size standard, thus correcting for lane-to-lane variation in electrophoretic distance. Depending on the allele: reference strand combination, peaks with an apparent base pair size of between ± 0.25 and $\pm 1.0\%$ of the clone DNA heteroduplex were scored as positive. The variable threshold for peak qualification is necessary because certain allele: reference-strand heteroduplexes exhibit greater intrinsic variation in their mobilities. After automatic scoring by DAX, a list of peak matches was imported into Microsoft Excel (Microsoft). Animals that scored positive for a given allele in all three reference strands were automatically considered positive for that allele. Fragment profiles from animals that scored positive in two of three reference strands for a particular allele were manually reexamined for peaks that may have been missed by the automatic peak detection (e.g., shoulder peaks adjacent to large, primary peaks).

To estimate the total number of Mauritian Cynomolgus macaque MHC class I alleles, we reexamined the RSCA data from all three reference strands, grouping the heteroduplex peaks from all 56 animals by apparent nucleotide size. Peaks with an apex intensity 20-fold greater than background were used in this analysis. Those peaks whose mobilities differed from one another by <0.5% were categorized as belonging to a single allele.

Determination of MHC I cell surface expression

1D-IEF separates the entire complement of expressed MHC I alleles on the basis of differential endpoint migration of individual α -chains in a discontinuous pH gradient within a polyacrylamide gel. The procedure was performed essentially as described in Watkins et al. (34). A stable transfectant of Mafa-B*440101 was produced in 721.221 cells as described previously (35). B lymphoblastoid cell lines were derived from Cynomolgus macaque PBMCs and from the Mafa-B*440101 transfectant. A total of 2×10^7 cells/sample was metabolically labeled with ^{35}S . The labeled cells were lysed and immunoprecipitated with w6/32 Ab. The immunoprecipitates were split into two fractions, and half were treated with neuraminidase type VIII to digest sialic and neuraminic acid moieties that decorate MHC molecules on the cell surface. The immunoprecipitates were loaded on a pH discontinuous acrylamide gel for isoelectric focusing. The gels were dried and MHC class I signal was detected by autoradiography following a 3- to 7-day exposure to film.

The alleles Mafa-A*230101, -A*350101, and -B*310101 are identical to Indian rhesus alleles Mamu-A*11, -A*13, and -B*01, respectively. We do not currently know whether these matches are due to subtle experimental contamination with rhesus samples or true *trans*-species allele sharing.

Results

Identification of 66 novel MHC I alleles in Cynomolgus macaques

MHC class I alleles identified in Rhesus macaques share significant homology at both the 5' and 3' termini. Therefore, we designed primers against these regions to amplify nearly full-length MHC I alleles from cDNA of Cynomolgus macaques. The PCR primers were broadly cross-reactive and successfully amplified MHC I alleles from Rhesus macaque, Cynomolgus macaque, Pig-tailed macaque (*Macaca nemestrina*), and human cDNA (data not shown). The PCR amplifications used the high-fidelity DNA polymerase Phusion, few amplification cycles (<25), and brief primer annealing steps (1 s) to minimize the formation of PCR amplification artifacts (30).

Between 48 and 192 individual amplicons/animal were cloned and sequenced. At least eight macaques were examined from each origin. We operationally defined MHC I alleles on the basis of at least two complete, identical sequences, although most alleles were found in at least three clones (36). Sixty-six MHC I alleles were identified. Thirty-five of these alleles were homologues of Rhesus MHC I A locus alleles, and 31 were MHC I B locus homologues. We adopted a modified nomenclature for naming the newly discovered alleles that prioritizes amino acid identity in the $\alpha 1$ and $\alpha 2$ domains that control peptide binding and TCR recognition (37). Although previous nonhuman primate MHC I nomenclatures are based either on ad hoc, arbitrary designations of sequence novelty or concordance with serological data (25, 27, 38), the goal of the new nomenclature is to cluster alleles with identical peptide-binding domains within the same top-level domain. In this nomenclature, any nonsynonymous variation in the sequence encoding the $\alpha 1$ and $\alpha 2$ domains is given a new top-level designation (e.g., Mafa-A*150101 vs Mafa-A*200101). Alleles that have nonsynonymous sequence variation outside of the regions encoding $\alpha 1$ and $\alpha 2$, but sequence identity within $\alpha 1$ and $\alpha 2$ are given a new subgroup designation (e.g., Mafa-A*150101 vs Mafa-A*150201) while any synonymous variants of a given allele are sub-subgrouped (Mafa-A*150101 vs Mafa-A*150102). By naming alleles in this way, related alleles with identical peptide-binding specificities are grouped.

MHC I allele sharing in Cynomolgus macaques from different origins

We identified 35 MHC I alleles in Vietnamese origin macaques, 25 in Chinese origin macaques, and 17 in Mauritian origin macaques. None of the alleles identified by Uda et al. (25, 26) were found in animals from these three origins. Moreover, there were no overlapping alleles present in animals from all three origins that we tested. The alleles Mafa-A*170101, -A*380101, -B*290101, -B*310101, -B*360101, -B*380101, -B*380301, -B*400101, and -B*410101 were found in both Vietnamese and Chinese origin macaques, while only Mafa-A*250201 was found in both Chinese and Mauritian origin macaques (Table I). Vietnamese and Mauritian origin macaques did not share any MHC class I alleles. From this analysis, we conclude that the MHC I allele repertoires of Cynomolgus macaques are largely origin specific.

We then reconstructed the phylogeny of the MHC I allele sequences. As expected, the MHC I A and B locus sequences form the two dominant branches of the tree. Within these branches, however, sequences from Vietnamese, Chinese, and Mauritian Cynomolgus macaques are interspersed (Fig. 1). This supports the hypothesis that these subpopulations have sampled diversity from a highly diverse ancestral population.

Preliminary identification of common MHC class I alleles

Common MHC class I alleles, analogous to the Mamu-A*01 allele in Indian Rhesus macaques, will be valuable in the development of Cynomolgus macaques as alternative nonhuman primate models for AIDS. Approximately 20% of captive bred Indian Rhesus macaques express Mamu-A*01. We are particularly interested in common MHC class I alleles that exist in more than one regional population and alleles that are exceptionally common within a single population. Twenty-five alleles were found in two or more of the Cynomolgus macaques studied. Of these 25 alleles, five were found in multiple animals from different origins. An additional 6 alleles were present in at least three animals from a single population. A subset of these alleles, Mafa-A*250201, -B*430101, -B*440101, and -B*460101, were identified in >50% of the animals examined. Remarkably, all of these alleles were found in Mauritian Cynomolgus macaques (Table II). Mafa-B*430101, -B*440101, and -B*460101 are consistently detected in combination; the same five animals possessed all three of these alleles.

High-resolution MHC I genotyping of Mauritian origin Cynomolgus macaques

We then determined the frequency of the common MHC I alleles in a cohort of 48 additional Mauritian origin macaques. It is difficult to develop allele-specific primers for allele-specific PCR in the absence of a reasonably complete allele database. Therefore, we used RSCA for genotyping (39). RSCA assesses the unique heteroduplex conformation that is assumed by a sequence-mismatched MHC I allele and a fluorescently labeled reference strand. A critical advantage of this method is that every heteroduplex will have a characteristic mobility signature. Although multiple alleles can potentially form identically migrating heteroduplexes with a single reference strand, it is unlikely that these migration similarities will be maintained if different reference strands are used. Therefore, increasing the number of reference strands improves the reliability of RSCA interpretation. Each RSCA experiment was internally controlled by including heteroduplexes of individual allele clones along with amplified cDNA from the eight animals used for allele identification. Three RSCA genotyping for the common alleles Mafa-B*430101, -B*440101, and -B*460101, as well as for the less common alleles -B*470101 and -B*510101,

Table I. Distribution of MHC I alleles in *Cynomolgus* macaques from different origins

Allele ^a	Uda et al. (origin unknown)	Vietnam	China	Mauritius
(Mafa-A*01)	X			
(Mafa-A*02)	X			
(Mafa-A*03)	X			
(Mafa-A*04)	X			
(Mafa-A*05)	X			
(Mafa-A*06)	X			
(Mafa-A*07)	X			
(Mafa-A*08)	X			
(Mafa-A*09)	X			
(Mafa-A*10)	X			
(Mafa-A*11)	X			
(Mafa-A*12)	X			
(Mafa-A*13)	X			
(Mafa-A*14)	X			
A*150101		X		
A*160101		X		
A*170101		X	X	
A*180101		X		
A*190101		X		
A*190201		X		
A*190301		X		
A*200101		X		
A*210101		X		
A*220101		X		
A*230101		X		
A*240101		X		
A*250101		X		
A*250201			X	X
A*260101		X		
A*260201		X		
A*270101		X		
A*280101			X	
A*290101				X
A*300101				X
A*310101				X
A*320101				X
A*330101				X
A*340101		X		
A*350101		X		
A*360101		X		
A*370101			X	
A*380101		X	X	
A*400101			X	
A*410101			X	
A*420101			X	
A*430101			X	
A*440101			X	
A*450101			X	
A*460101			X	
(Mafa-B*01)	X			
(Mafa-B*02)	X			
(Mafa-B*03)	X			
(Mafa-B*04)	X			
(Mafa-B*05)	X			
(Mafa-B*06)	X			
(Mafa-B*07)	X			
(Mafa-B*08)	X			
(Mafa-B*09)	X			
(Mafa-B*10)	X			
(Mafa-B*11)	X			
(Mafa-B*12)	X			
(Mafa-B*13)	X			
(Mafa-B*14)	X			
(Mafa-B*15)	X			
(Mafa-B*16)	X			
(Mafa-B*17)	X			
(Mafa-B*18)	X			
(Mafa-B*19)	X			
(Mafa-B*20)	X			
(Mafa-B*21)	X			
(Mafa-B*22)	X			
(Mafa-B*23)	X			
(Mafa-B*24)	X			
(Mafa-B*25)	X			
(Mafa-B*26)	X			
B*270101		X		
B*280101		X		
B*290101		X	X	
B*300101		X		

(Table continues)

Table I. Continued

Allele ^a	Uda et al. (origin unknown)	Vietnam	China	Mauritius
B*310101		X	X	
B*320101		X		
B*330101		X		
B*340101		X		
B*350101		X		
B*360101		X	X	
B*370101		X		
B*380101		X	X	
B*380201			X	
B*380301		X	X	
B*390101		X		
B*400101		X	X	
B*410101		X	X	
B*420101			X	
B*430101				X
B*440101				X
B*440201				X
B*450101				X
B*460101				X
B*470101				X
B*470201				X
B*480101				X
B*490101				X
B*500101				X
B*510101				X
B*520101			X	
B*530101			X	

^a Uda et al. alleles were described in Ref. (25, 26)

matched the typing obtained by sequencing individual MHC clones (Fig. 2). The exceptionally high frequencies of *Mafa-B*430101*, *-B*440101*, and *-B*460101* were verified in the additional 48 animals. More than 50% of the animals possessed *Mafa-B*430101* (59%), *-B*440101* (63%), and *-B*460101* (53%). Moreover, the combination of *Mafa-B*430101*, *-B*440101*, and *-B*460101* was found in 52% of the animals. The alleles *Mafa-B*470101* and *Mafa-B*510101* were found in 20 and 29% of the animals, respectively (Fig. 3). Therefore, we conclude that extraordinary MHC I allele sharing is a characteristic feature of Mauritian *Cynomolgus* macaques.

Estimation of MHC I allele transcript repertoire of Mauritian *Cynomolgus* macaques

The previous analyses measured the frequency and sequence of transcribed MHC I alleles. There is still considerable uncertainty about the expression of macaque MHC I genes. Sequencing of the complete Rhesus MHC region identified >20 potentially active MHC I genes, but it appears that only a subset of these genes are transcribed (40). Furthermore, it is possible that only a subset of the transcribed genes are translated, or that many of the transcribed genes are expressed at very low levels (23).

Fewer total MHC class I alleles were identified in Mauritian *Cynomolgus* macaques than in either Chinese or Vietnamese animals, but it is unlikely that our approach uncovered all of the alleles given in each population. We estimated the sensitivity of allele discovery by examining how many heteroduplex peaks are present in each animal's RSCA profile. In the eight Mauritian *Cynomolgus* macaques for which both clone sequence and RSCA are available, an average of 10.1 heteroduplex peaks were identified by RSCA, while an average of only 5.3 alleles were identified by clone sequencing.

How many alleles would be present in a complete Mauritian *Cynomolgus* macaque MHC I database? We counted the total number of unique RSCA heteroduplex signatures in the 56 genotyped macaques. A total of 37, 36, and 39 heteroduplex peaks

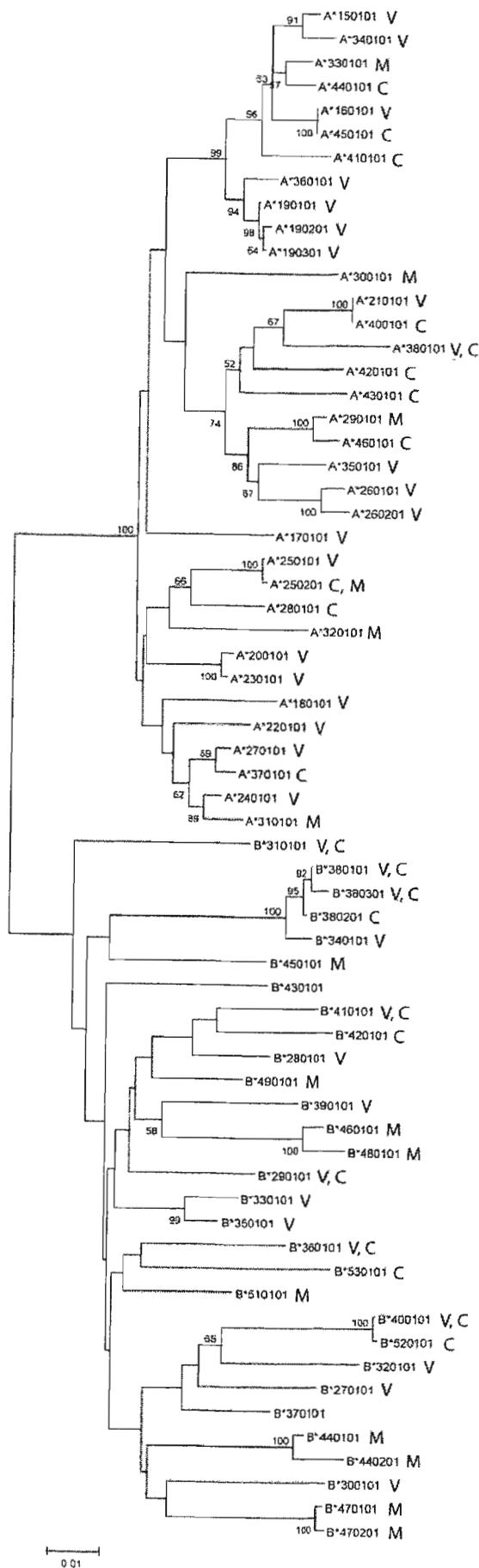


Table II. Twenty-five MHC I alleles detected in multiple *Cynomolgus macaques*

Allele	Vietnam ^a	China ^a	Mauritius ^a
A*170101	2/11	1/8	0/8
A*180101	4/11	0/8	0/8
A*190101	1/11	1/8	0/8
A*250201 ²	0/11	1/8	5/8
A*290101	0/11	0/8	3/8
A*300101	0/11	0/8	2/8
A*310101	0/11	0/8	2/8
A*380101	1/11	2/8	0/8
B*290101	2/11	1/8	0/8
B*310101	1/11	1/8	0/8
B*360101	2/11	3/8	0/8
B*330101	1/11	1/8	0/8
B*380301	1/11	1/8	0/8
B*390101	2/11	0/8	0/8
B*400101	2/11	2/8	0/8
B*410101	1/11	1/8	0/8
B*430101	0/11	0/8	5/8
B*440101	0/11	0/8	5/8
B*440201	0/11	0/8	2/8
B*450101	0/11	0/8	2/8
B*460101	0/11	0/8	5/8
B*470101	0/11	0/8	2/8
B*490101	0/11	0/8	2/8
B*510101	0/11	0/8	2/8
B*530101	0/11	2/8	0/8

^a Number of animals with MHC class I allele sequence divided by the total number of animals examined from each cohort.

^b Shading denotes alleles found in >50% of animals studied.

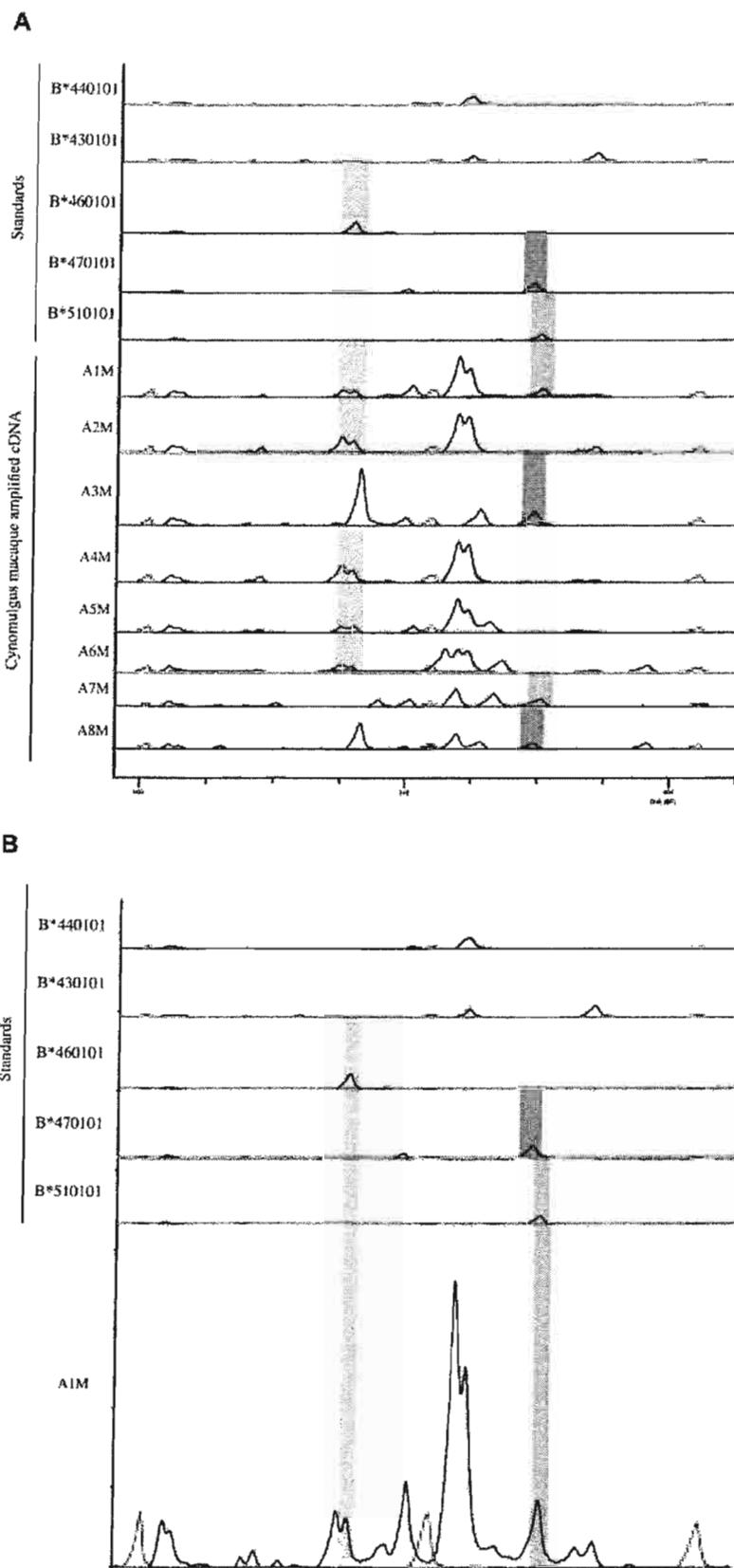
formed with the Mafa-A*200101, Mamu-B*07, and Mafa-B*370101 reference strands, respectively. Therefore, we estimate that there are ~40 total MHC I alleles in Mauritian *Cynomolgus macaques*. Our estimate, however, is based on a sample of only 56 animals. Additional alleles may be revealed if a larger population of animals is analyzed.

MHC I expression in Mauritian *Cynomolgus Macaques*

If the common MHC I genes in Mauritian *Cynomolgus macaques* are not expressed, the value of these animals may be diminished. To address this issue, we transfected a MHC I-null B cell line (721.221) with a clone containing the sequence of Mafa-B*440101. We detected expression of Mafa-B*440101 by surface staining the transfectant with a pan-MHC class I Ab (W6/32) conjugated to FITC. Expression of Mafa-B*440101 in Mauritian *Cynomolgus macaques* was verified by immunoprecipitating MHC I proteins from the surface of seven immortalized B cell lines and separating the immunoprecipitated proteins by 1D-IEF. The Mafa-B*440101 signature from the transfectant is present in the MHC molecules precipitated from A1M, A2M, A4M, A5M, and A6M cell lines, in both the presence and absence of neuraminidase (Fig. 4). These same animals were Mafa-B*440101-positive by both RSCA and individual clone sequencing. Though a band with a slightly more acidic isoelectric point is also observed in the neuraminidase-treated sample Mafa-B*440101-negative animal A8M, the banding pattern differs in the neuraminidase-untreated samples. Although we do not currently have MHC I transfectants for the

FIGURE 1. Neighbor-joining tree of 66 newly discovered *Cynomolgus macaque* MHC I alleles. The numbers on the branches are percentages of 1000 bootstrap samples supporting the branch; only values >50 are shown. The subpopulations of *Cynomolgus macaques* possessing each allele are shown to the right of each alleles, with C = China; V = Vietnam; M = Mauritius.

FIGURE 2. Concordance between MHC I typing by cloning and sequencing and RSCA in Mauritian origin Cynomolgus macaques. **A**, RSCA was performed on amplified cDNA from the eight Mauritian Cynomolgus macaques used for allele discovery, as well as on amplified DNA clones of five MHC I B locus alleles. Each lane contained an internal size standard (red peaks) to normalize intragel variation in sample mobility. All heteroduplexes were formed with 6FAM-labeled Mafa-A*200101 reference strand. Colored boxes indicate concordance between genotyping by RSCA and the results obtained from amplified cDNA cloning and sequencing. **B**, It is difficult to resolve the MHC I allele positivity for *Mafa-B*430101* because of the scaling of the plots. Here, we present a subset of the RSCA data from **A** to illustrate that magnification of the y-axis allows for the ready identification of the *Mafa-B** peak, as well as peaks corresponding to three other MHC I alleles. To generate the display image, the y-axis of the AIM trace was mathematically transformed by multiplying the signal by a factor of 25. Without mathematical transformation, the signal from the DNA clones is typically several-fold higher than the signal from the amplified cDNA samples. The small secondary peak in the *Mafa-B** clone is the result of signal bleedthrough from an exceptionally strong *Mafa-B*440101* heteroduplex signal. No signal bleedthrough was observed in amplified cDNA samples (data not shown).



other common alleles, at least two additional bands are shared between these animals, suggesting that at least two more of the common alleles are expressed (Fig. 4). We also calculated the isoelectric point for each of the common MHC class I alleles from

its translated nucleotide sequence (protein calculator; C. Putnam, The Scripps Research Institute, La Jolla, CA) and found that *Mafa-B*440101* had the most basic isoelectric point, in agreement with the position of this protein in the focusing matrix.

Animal	B*430101	B*440101	B*460101	B*470101	B*510101	Animal	B*430101	B*440101	B*460101	B*470101	B*510101
235						73-116					
227						63-100					
209						93-340					
106						103-108					
207						161					
205						131					
117						156					
230						176					
213						197					
212						122					
110						175					
232						157					
225						189					
103						204					
220						154					
219						236					
73-106						199					
39-349						169					
73-101						187					
103-107						166					
73-105						A1M					
103-114						A2M					
73-108						A3M					
73-118						A4M					
63-65						A5M					
63-73						A6M					
63-104						A7M					
63-59						A8M					

FIGURE 3. Genotyping of 56 Mauritian origin *Cynomolgus* macaques for common MHC I alleles by RSCA. Amplified cDNA from each animal was subjected to RSCA with three different MHC I-derived reference strands. RSCA was scored as described in *Materials and Methods*. A positive allele genotype requires the presence of a characteristic allelic heteroduplex in all three of the tested reference strands.

Discussion

Nonhuman primates are widely used in biomedical research. AIDS research, in particular, relies on the availability of Rhesus macaques to study preclinical vaccine efficacy and disease pathogenesis. Trials that focus on cellular immunity or use reduction in viral burden as an end point exacerbate the Rhesus macaque shortage by selectively using animals possessing MHC I alleles, such as Mamu-A*01, that bind known CD8⁺ T lymphocyte epitopes (41). Less than 25% of Indian Rhesus macaques are Mamu-A*01 positive (9), so these trials require a pool of available animals four-fold larger than the study size. In other words, a two-armed vaccine trial with five vaccinees and five vaccine naive Mamu-A*01-positive Indian Rhesus macaques requires an effective cohort size of at least 40 animals. To relieve the demand on Indian origin Rhesus macaques, we characterized MHC class I alleles from *Cynomolgus* macaques. We determined that *Cynomolgus* macaques from different origins have largely independent MHC class I allele repertoires. Several MHC I alleles were found in animals from two populations and may be attractive targets for future research.

We speculate that MHC I allele repertoire diversity may be a general feature of regional subpopulations of macaques. Anecdotal support for this speculation is found in the observation that the extremely common Indian Rhesus macaque MHC I allele Mamu-A*01(10) is not detected in Chinese Rhesus macaques (20). This result, coupled with our findings, may provide an impetus for the reporting of macaque origins in pathogenesis studies and spur research into the functional consequences of these regional differences. Currently, macaque origins are rarely specified in methods

sections of immunology and pathogenesis publications (42–54), though this data may be profoundly influential.

Importantly, we found that Mauritian origin *Cynomolgus* macaques possess multiple extremely common MHC I alleles, with >50% of animals having the allele combination *Mafa-B*430101*, *-B*440101*, and *-B*460101*. To our knowledge, these are the highest-frequency MHC I alleles ever described in a population of macaques.

Mauritian *Cynomolgus* macaques may represent an extraordinarily valuable resource for pathogen research. Because the MHC loci are among the most polymorphic in primate genomes (55), it is reasonable to predict that non-MHC genetics in these animals may also be simplified relative to Asian origin macaques. This may be important, as HIV disease progression is likely to be influenced by polymorphisms in other loci, such as Kir receptors (56, 57), cytokine receptors and their ligands (58), in addition to MHC products (59). Vaccine trials that rely on comparisons between groups stand to benefit from the availability of animals with reduced genetic variability. Moreover, mapping cellular immune responses bound by common MHC I alleles will facilitate vaccine evaluation and studies of SIV pathogenesis.

The unusual degree of allele sharing in Mauritian *Cynomolgus* macaques is predictable given the natural history of this population. Anthropological and historical evidence suggests that the macaques were introduced to the island ~400 years ago by European seafarers (60). mtDNA analysis of Mauritian origin macaques supports the existence of a population bottleneck at the time of introduction; in fact, the founding population may have included only a single female (61). Although low mtDNA divergence does not

A

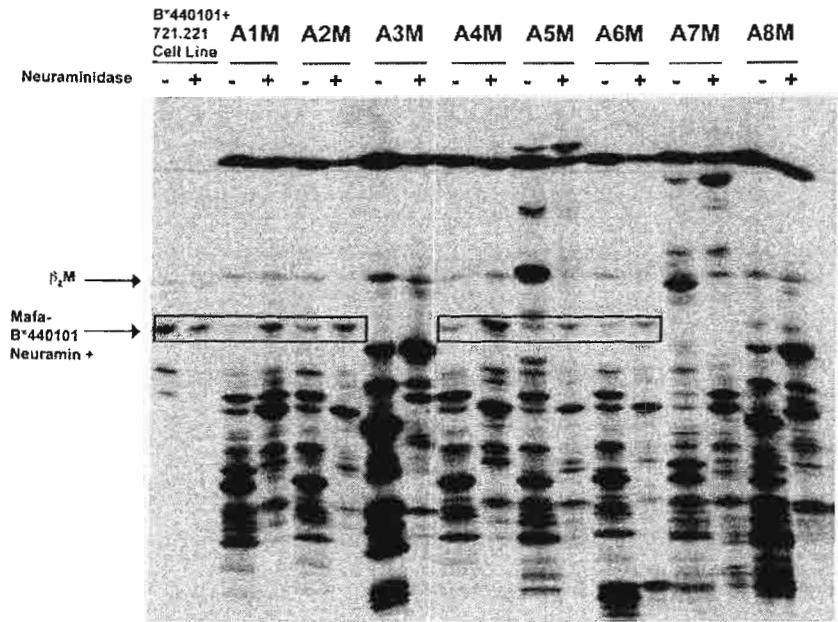
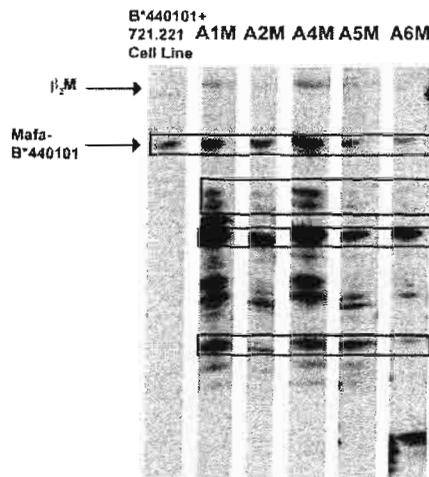


FIGURE 4. 1D-IEF of immunoprecipitated MHC I molecules verifies expression of Mafa-B*440101 and suggests shared expression of other common alleles. *A*, 1D-IEF was performed with a 721.221 cell line stably transfected with Mafa-B*440101 and B lymphoblastoid cell line from animals A1M–A8M in the presence and in the absence of neuraminidase. The band corresponding to Mafa-B*440101 in the neuraminidase-treated samples is boxed. *B*, Simplified view of ID-IEF data from *A* showing the neuraminidase treated samples from animals A1M, A2M, A4M, A5M, and A6M. These animals share at least four alleles by RNA genotyping. IEF bands common to each of these animals are boxed.

B



necessarily correlate with low nuclear gene variability, other studies have found the genetic diversity of nuclear genes in this population is lower than in Asian origin *Cynomolgus* macaques (62). Additionally, a recent study of MHC II sequences detected 20 Mafa-DRB sequences in a survey of 10 Chinese origin *Cynomolgus* macaques, while only 15 Mafa-DRB sequences were found in a population of 58 Mauritian origin macaques (63).

A significant unanswered question is whether the Mauritian *Cynomolgus* macaques we studied are representative of the entire population on the island. We do not have any information on the heritage of the animals that we studied because the blood samples were purchased commercially. It may be informative to study MHC haplotypes with microsatellite DNA markers, as recently described in Rhesus macaques (64), to infer the relationships among animals and to define the region configurations of common MHC I haplotypes. It is possible that the *Mafa-B*430101*, *-B*440101*, *-B*460101* alleles are linked on a frequent MHC haplotype.

We do know, however, that the animals used in this study were wild-caught and, thus, unlikely to be related. Additionally, the blood samples were obtained on two different occasions, further increasing the likelihood that the results are extensible to the general population of Mauritian macaques. *Cynomolgus* macaques, as a species, are highly adaptable and can thrive in a variety of ecological niches, particularly those disrupted by human activity (60). Mauritius is <2000 km (2), with unequal monkey distribution throughout the small island. Previous analysis of mtDNA using monkeys captured from different sites did not observe genetic stratification by habitat, providing anecdotal evidence that subpopulations of macaques on the island are genetically homogenous (61).

Do Mauritian origin *Cynomolgus* macaques represent a reasonable alternative animal model for AIDS research? More than 3000 Mauritian *Cynomolgus* macaques are imported for research yearly (T. Demarcus, unpublished observations), eclipsing the number of *Cynomolgus* macaques imported from any other country. How does this compare with the demand for Rhesus macaques in AIDS

vaccine research? According to the HIV/SIV Vaccine Trials Database, a total of 332 Rhesus macaques was used in 18 vaccine trials published in 2004. Even if only a fraction of all ongoing vaccine trials generate publishable data during a single year, a sizable population of these Mauritian Cynomolgus macaques should be available for vaccine projects that require genetically defined animals.

For the value of this model to be realized, Mafa-B*430101, -B*440101, -B*460101-positive animals must be susceptible to pathogenic SIV infections, without exhibiting the unusual resistance associated with certain MHC class I alleles in Indian Rhesus macaques (15–17). Previous work has shown that Cynomolgus macaques generally (65, 66), and Mauritian origin Cynomolgus macaques in particular (67–69), can be infected with pathogenic SIV and develop sAIDS. When compared with Indian Rhesus macaques, Cynomolgus macaques require a higher dose of virus to consistently establish SIV infection (68) and maintain lower viral loads more similar to HIV-infected humans (66). This could complicate the evaluation of vaccines that seek to reduce set point viral load because differences between vaccinees and naive controls may be more difficult to detect. Conversely, the exceptionally high viral loads witnessed in Indian Rhesus macaques may represent an unnaturally high barrier for vaccination, and may be the consequence of deriving viral strains by serial passage of SIV through multiple Rhesus macaques (70). It may be possible to recapitulate high viral loads in Cynomolgus macaques, if desired, by serial passage.

A related concern is whether the common MHC I alleles are likely to bind and present SIV-derived peptides to CD8⁺ T-lymphocytes. ID-IEF confirms that Mafa-B*440101 is expressed, and strongly suggests the expression of at least two additional, common MHC I alleles. What is the likelihood that expressed MHC I alleles restrict SIV-specific immune responses? In the Indian Rhesus macaque, epitope panning has been performed on four MHC I alleles. In these studies, the peptide binding motif for an allele is determined; the SIV proteome is scanned for peptides that fit the motif; the peptides are experimentally tested for binding affinity; and peptides that exceed the binding threshold are tested for antigenicity in SIV-infected animals. For each allele, at least five SIV-specific CD8⁺ T lymphocyte responses were identified (41, 71, 72). Therefore, it is reasonable to forecast that at least one (and perhaps all) of the three common MHC I alleles identified in Mauritian Cynomolgus macaques will restrict SIV-specific CD8⁺ T lymphocyte responses.

In summary, we have established the foundation for performing MHC I-dependent research projects in Cynomolgus macaques. We show that macaques from different origins have largely independent MHC I allele repertoires and should not be used interchangeably for studies that may be influenced by MHC I genetics. Mauritian origin animals may be uniquely valuable for this type of research because of their atypical and unprecedented MHC I repertoire: >50% of these animals share a combination of three alleles.

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Disclosures

The authors have no financial conflict of interest.

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A review of background findings in cynomolgus monkeys (*Macaca fascicularis*) from three different geographical origins

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Abstract

This review was performed to assess variations in background observations in cynomolgus macaques (*Macaca fascicularis*) originating from three breeding centres located in Mauritius, The Philippines and Vietnam. The data and tissue samples from 90 cynomolgus monkeys (approximately evenly distributed between the three sources) comprising the control groups from 11 regulatory toxicology studies were used for this investigation. Clinical data – age, body weight, organ weights, haematology and serum biochemistry – were analyzed. Samples of stomach, colon, kidney, heart, liver, spleen and lung were examined microscopically and graded to characterize the degree of lymphoplasmacytic cell infiltration.

The main microscopic origin-related variations concerned the digestive tract, where the lymphoplasmacytic cell infiltration grade was significantly lower ($p \leq 0.001$) in cynomolgus monkeys from Mauritius when compared with those from Asia. Generally, only the antral mucosa of the stomach was infiltrated in cynomolgus monkeys from The Philippines, whereas both the fundic and antral regions were infiltrated in those from Vietnam. The digestive tract infiltration grade was strongly correlated with the mean white blood cell count in monkeys from all three sources. Spiral-shaped bacteria were observed in the stomach of monkeys from all three sources, but their presence did not correlate with the severity of the gastric infiltrate. *Helicobacter heilmannii*-type bacteria were almost always seen in the fundus, *Helicobacter pylori*-type bacteria were only occasionally seen in the antral region.

The incidences of other microscopic findings, such as urothelial cytoplasmic inclusions or *Balantidium coli* in the caecum, also varied according to the source of the monkeys. Some variations in relative organ weights, haematology and serum biochemistry were also related to the origin of the monkeys, but these did not correlate with the microscopic findings.

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Introduction

The cynomolgus macaque (long-tailed or crab-eating macaque) *Macaca fascicularis* is one of the most widely used non-human primate (NHP) species in biomedical research. Due to the lack of availability and relative expense of rhesus monkeys, the cynomolgus macaque

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has replaced the rhesus in areas such as applied drug development, toxicology and teratology (Hearn, 2002). Originating from Southeast Asia, these animals are now purpose-bred in a limited number of breeding centres in different geographical locations. As there are very few breeding facilities in Europe, most of European countries totally depend on NHP importation. Our contract research organization uses cynomolgus macaques provided by three suppliers. In our routine toxicological studies, varying background changes were noted in animals from these three origins. These variations concerned mainly the extent of lymphoplasmacytic cell infiltrates in several organs.

The aim of this review was to compare untreated cynomolgus macaques from the three major suppliers used by MDS and to investigate the background variations of biochemical and haematological data as well as final body weight, organ weights and microscopic evaluation of organs.

Materials and methods

Cynomolgus macaques (*M. fascicularis*)

Ninety purpose-bred cynomolgus macaques were selected from the control groups of 11 studies. The studies were conducted in compliance with European regulations governing the housing and use of animals in an AAALAC-accredited facility. The monkeys were obtained from three different suppliers, as given in Table 1. The age of each monkey in months was provided by the supplier.

All of the breeding facilities had been audited by MDS and found to have an acceptable sanitary status. The cynomolgus monkeys from Mauritius did not undergo any serology analyses during the exportation quarantine since the island is free from rabies, B herpes virus and retroviruses. Those from The Philippines were tested for rabies and herpes B during quarantine. Vietnamese macaques were tested for rabies, herpes B, simian immunodeficiency virus (SIV), simian type-D retroviruses (SRV/D) and filovirus. All animals had tuberculosis tests and coproscopy examinations for bacteria and parasites at the breeding facilities and

again after arrival at MDS. They were also treated against internal parasites before allocation to a study.

An acclimatization period was set with a minimum of 6 weeks between arrival and start of treatment and a minimum period of 3 weeks acclimatization to the study housing conditions.

Clinical and necropsy examinations

Haematology and serum clinical biochemistry values were obtained by blood sampling on conscious animals the day of the terminal sacrifice. Terminal body weight and selected relative organ weights were recorded for each animal during the necropsy procedure. The data were first analyzed using a one-way ANOVA to detect any differences between the groups of animals from different suppliers. A Newman-Keuls test was then performed for parameters with a significant ($p \leq 0.05$) *F* statistic.

Microscopic examination

The initial evaluation focused on the digestive tract (stomach, duodenum, colon and caecum), kidneys, heart, liver, spleen and lungs. The lymph nodes were also examined, but the individual variation precluded an intergroup analysis. The small intestine examination was performed only on the duodenum because of strong similarities between the three segments. The microscopic slides were obtained after a 10% buffered formalin fixation, paraffine wax embedding, 4–6 μ m sections, and haematoxylin–eosin (HE) stain.

The recorded microscopic findings from in study data were first evaluated. A preliminary step consisted of harmonizing the various terms used by different pathologists. There were differences in the use of more diagnostic terms such as gastritis, nephritis, pneumonia, hepatitis, etc. versus more descriptive terminology, e.g. lymphoplasmacytic, mononuclear or lymphoid cell infiltration.

All microscopic findings were first listed from the selected organs. Special stains such as Masson trichrome and Congo Red were carried out when necessary. The tissues from the selected organs were then re-examined and graded according to their degree of lymphoplasma-

TABLE 1. Origin of cynomolgus monkeys (*Mucaca fascicularis*)

Geographical origin	Supplier	Number of studies	Number of animals
Mauritius	Noveprim group	5	30
The Philippines	Siconbrec	3	28
Vietnam	Nafovanny	3	32

Noveprim Group (C.R.P. Le Vallon), Ferny S.E., Mahebourg, Ile Maurice.

Siconbrec: So. Payong, Brgy. T. Kutyo, Tanay Rizal 1980, The Philippines.

Nafovamy, Tam Phuoc Hamlet, Long Thanh District, Dong Nai Province, Vietnam.

cytic cell infiltrations (LCIs). Three segments were graded for the digestive tract: the gastric fundic mucosa, the gastric antral mucosa and the colon. The severity of the infiltration in the duodenum was very similar to that in the gastric antrum and was therefore not recorded. The grade 0 corresponded to the expected background level and grades 1–5 corresponded to minimal, slight, moderate, marked and severe increases of cellularity, respectively. Mean values for each animal were then calculated and used to compare the monkeys from the three sources. The differences were analyzed by a Kruskal–Wallis test with origin as a factor, followed by Wilcoxon test in case of significant Kruskal–Wallis result.

The individual clinical data were then evaluated to determine any correlation with the histological findings, using linear regression.

Results

Age

There were no significant differences between the ages of the male and female monkeys. Ages ranged from 21 to 37 months with a mean value of 28.5 months. Monkeys of 19–31 months were considered juveniles (no sexual dimorphism). Those of 32–44 months were considered adolescent (not yet sexually mature). Confirmed sexual maturity was only reached at 4 years of age for the females and 6 years for the males.

Age ranges varied from one supplier to another as the mean values were 30.3, 30.7 and 24.8 months for animals from Mauritius, The Philippines and Vietnam, respectively. Vietnamese monkeys were significantly younger than those from the two other sources ($p \leq 0.001$).

Clinical and necropsy examinations

The terminal body weights were higher in males than in females, as indicated in Table 2. The animals originating from Vietnam weighed less than those from Mauritius ($p \leq 0.001$) and those from The Philippines

($p \leq 0.05$). The cynomolgus monkeys from Vietnam had lower relative kidneys, spleen and liver weights and a slightly higher relative heart weight. These organ weight variations were statistically significant only for the lower relative spleen weight in Vietnamese males and females when compared with the two other origins ($p \leq 0.001$), and for the lower relative kidney weight in Vietnamese females ($p \leq 0.01$).

As detailed in Table 3, the haematology values showed a significant difference between Vietnamese monkeys and those from the two other sources. The Vietnamese monkeys had fewer red blood cells ($p \leq 0.001$), which was compensated by a higher mean corpuscular volume ($p \leq 0.001$) and a higher mean corpuscular haemoglobin ($p \leq 0.001$) resulting in a greater haemoglobin concentration ($p \leq 0.05$). The mean total white blood cell count was lower in cynomolgus monkeys from Mauritius ($p \leq 0.05$), particularly in males. The eosinophilic granulocyte percentage was higher in cynomolgus monkeys from The Philippines ($p \leq 0.001$). The lymphocyte count was higher than neutrophil count, except for the Mauritian females.

Serum biochemical values varied between the different groups, as presented in Table 4. Mauritian cynomolgus monkeys had a higher serum calcium concentration ($p \leq 0.01$), and lower serum phosphorus ($p \leq 0.001$) than the monkeys from the two other sources. Serum creatinine values were lower in monkeys from The Philippines ($p \leq 0.001$) whereas there were no significant differences for urea. The hepatic enzymes also showed some significant variations: the serum alkaline phosphatase (ALP) activity was lower ($p \leq 0.05$) and ASAT was higher ($p \leq 0.001$) in the monkeys from Vietnam. ALAT was lower in the cynomolgus monkeys from The Philippines ($p \leq 0.05$).

Microscopic findings

Lymphoplasmacytic cell infiltrates in the selected organs: histologic features and grading

Origin-related differences in the grade of LCI per organ were present only in the digestive tract ($p \leq 0.001$), as presented in Table 5. There were no differences

Table 2. Mean terminal body weight and relative organ weight

Mean \pm SD	Males			Females		
	Mauritius	The Philippines	Vietnam	Mauritius	The Philippines	Vietnam
Body weight (g)	3054 \pm 725	2729 \pm 420	2270 \pm 301	2647 \pm 384	2474 \pm 240	2196 \pm 240
Relative organ weights (%)						
Kidneys	0.48 \pm 0.05	0.46 \pm 0.06	0.43 \pm 0.06	0.51 \pm 0.05	0.51 \pm 0.04	0.44 \pm 0.06
Liver	2.14 \pm 0.25	2.20 \pm 0.28	2.05 \pm 0.23	2.18 \pm 0.21	2.38 \pm 0.24	2.09 \pm 0.19
Spleen	0.21 \pm 0.05	0.25 \pm 0.03	0.14 \pm 0.03	0.23 \pm 0.04	0.26 \pm 0.05	0.14 \pm 0.03
Heart	0.37 \pm 0.03	0.36 \pm 0.03	0.37 \pm 0.04	0.36 \pm 0.05	0.34 \pm 0.04	0.38 \pm 0.03

Table 3. Mean haematological values

Mean \pm SD	Males			Females		
	Mauritius	The Philippines	Vietnam	Mauritius	The Philippines	Vietnam
RBC*** (M/mm ³)	6.53 \pm 0.50	6.91 \pm 0.24	5.82 \pm 0.31	6.44 \pm 0.77	6.64 \pm 0.51	5.58 \pm 0.39
Hb** (g/dL)	13.1 \pm 1.0	12.9 \pm 0.3	13.4 \pm 0.7	12.6 \pm 1.2	12.3 \pm 0.9	13.5 \pm 0.8
MCV*** (fL)	68.4 \pm 2.8	65.5 \pm 3.1	77.4 \pm 2.6	68.5 \pm 4.5	65.7 \pm 1.9	78.2 \pm 3.0
MCH*** (pg)	20.1 \pm 0.8	18.8 \pm 0.7	23.1 \pm 1.1	19.7 \pm 1.3	18.4 \pm 0.9	24.2 \pm 0.9
WBC** (k/mm ³)	9.44 \pm 2.83	13.79 \pm 3.62	11.02 \pm 2.76	10.30 \pm 2.65	11.15 \pm 3.73	12.40 \pm 3.54
L (%)	56.4 \pm 13.7	59.9 \pm 10.4	59.5 \pm 9.5	45.5 \pm 12.9	52.3 \pm 11.5	48.7 \pm 16.1
N (%)	36.5 \pm 14.7	31.5 \pm 11.1	35.2 \pm 9.9	47.3 \pm 13.4	37.5 \pm 11.9	46.0 \pm 17.3
E*** (%)	1.2 \pm 0.8	3.5 \pm 2.7	0.9 \pm 0.6	1.2 \pm 0.9	4.5 \pm 2.8	1.1 \pm 0.8

RBC: red blood cell count; Hb: haemoglobin; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; WBC: total white blood cell count; L: lymphocytes; N: neutrophils; E: eosinophils. */**/** parameters presenting significant differences using a one-way ANOVA test, with source as a factor. * $p \leq 0.05$. ** $p \leq 0.01$. *** $p \leq 0.001$.

Table 4. Mean serum biochemical values

Mean \pm SD	Males			Females		
	Mauritius	The Philippines	Vietnam	Mauritius	The Philippines	Vietnam
Urea (g/L)	0.48 \pm 0.14	0.49 \pm 0.07	0.49 \pm 0.12	0.52 \pm 0.14	0.48 \pm 0.08	0.44 \pm 0.13
Creat.*** (mg/L)	8.7 \pm 1.1	6.9 \pm 0.6	8.2 \pm 1.3	8.2 \pm 1.1	6.8 \pm 1.0	7.5 \pm 1.1
Ca** (mg/L)	104 \pm 6.1	97 \pm 2.7	100 \pm 6.1	104 \pm 7.2	101 \pm 7.7	99 \pm 4.1
P*** (mg/L)	65 \pm 8.3	74 \pm 11.3	73 \pm 7.5	58 \pm 11.9	73 \pm 8.9	72 \pm 9.4
Ca/P***	1.62 \pm 0.25	1.34 \pm 0.19	1.38 \pm 0.16	1.87 \pm 0.42	1.41 \pm 0.27	1.40 \pm 0.22
ALP** (IU/L)	1859 \pm 426	1887 \pm 323	1513 \pm 467	1550 \pm 379	1684 \pm 616	1340 \pm 357
ASAT*** (IU/L)	36 \pm 12	33 \pm 6	51 \pm 15	35 \pm 8	34 \pm 9	51 \pm 18
ALAT* (IU/L)	46 \pm 13	38 \pm 19	55 \pm 20	49 \pm 22	38 \pm 14	46 \pm 19

Creat.: creatinine; Ca: calcium; P: phosphorus; Ca/P: phosphocalcemic ratio; ALP: alkaline phosphatase; ASAT: aspartate aminotransferase; ALAT: alanine aminotransferase. */**/** parameters presenting significant differences using a one-way ANOVA test, with source as a factor. * $p \leq 0.05$. ** $p \leq 0.01$. *** $p \leq 0.001$.

Table 5. Mean lymphoplasmacytic cell infiltration grade in the digestive tract and comparison between the three origins

Digestive part	Mean infiltration grade (\pm SD)			Intergroup comparison (Wilcoxon test)		
	Mauritius	The Philippines	Vietnam	Mauritius vs. The Philippines	Mauritius vs. Vietnam	The Philippines vs. Vietnam
Gastric fundus	0.00 \pm 0.00	0.86 \pm 1.04	2.45 \pm 0.68	***	***	***
Gastric antrum	0.23 \pm 0.63	2.93 \pm 0.81	2.41 \pm 1.10	***	***	*
Colon	0.67 \pm 0.66	0.89 \pm 0.69	1.44 \pm 0.62	ns	***	**

ns = not significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

between the males and females. A distinct pattern of lymphoplasmacytic infiltration was reported for each of the organs examined, and was described as follows. The incidences of LCI for each origin are detailed in Table 6.

Digestive tract: Subacute to chronic gastritis was observed in 65/90 cynomolgus monkeys and was characterized by a diffuse lymphoplasmacytic infiltra-

tion of the lamina propria causing dissection of the gastric glands. Marked differences were noted according to the gastric location. The gastritis was marked and diffuse in the antral mucosa. It was moderate, more superficial and often patchy in the fundic mucosa (Fig. 1). A preferential location was noted in the transition zone between the fundic and the antral mucosa.

Table 6. Incidence table (%) of microscopic findings showing variations in control cynomolgus monkeys between the different origins

	Origin (number of monkeys)	M (30)	P (28)	V (32)
Digestive tract	Stomach: LPCI/chronic diffuse gastritis	17	100	100
	Colon: LPCI/chronic diffuse colitis	57	71	97
	Caecum/colon: <i>Balantidium coli</i> in the lumen	13	75	66
Kidney	LPCI/interstitial nephritis, focal, chronic	43	61	75
	Cytoplasmic inclusions in the urothelium	90	100	31
Heart	LPCI	40	50	63
Liver	LPCI	73	75	69
Spleen	Increased cellularity of white pulp	40	54	50
Lung	LPCI/pneumonitis, focal, chronic	3	14	19

M: Mauritius. P: The Philippines. V: Vietnam, LPCI: lymphoplasmacytic cell infiltration.

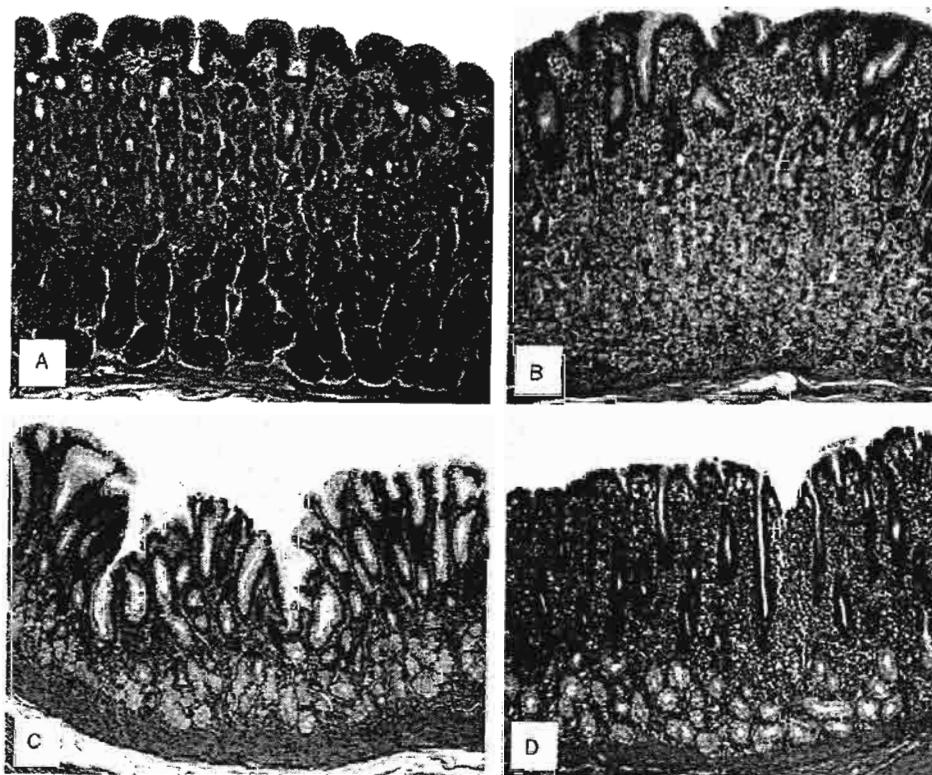


Fig. 1. Cynomolgus stomach. Lymphoplasmacytic cell infiltration. Fundic part of the gastric mucosa with (A) non-increased cellularity and (B) moderately increased cellularity. In the antral part of the gastric mucosa (C) non-increased cellularity and (D) markedly increased cellularity. HE stain. $\times 40$.

In some animals, there were also evidence of active inflammation, i.e. multifocal haemorrhage, and neutrophils in glands (crypt abscesses) or in the surface epithelium. In addition to the diffuse infiltrate, prominent lymphoid aggregates were observed within the deep lamina propria, forming primary or secondary lymphoid follicle and displacing the gastric glands. These were generally located just above the muscularis mucosa or

sometimes more superficially in the lamina propria. These lymphoid nodules were more frequent in the antral region than in the fundus. They were on occasions more prominent when there was a concurrent gastritis, but there was no correlation with the grading of the lymphoplasmacytic infiltration in the lamina propria.

Other histologic features – such as glandular cystic dilatation, glandular atrophy and regenerative change

(intestinal metaplasia) - were occasionally seen accompanying the chronic gastritis.

The duodenum most often presented a similar infiltration grade as the antral part of the stomach. Subacute to chronic colitis and typhlitis were also observed, sharing the same histologic features as those of the gastritis but with varying severity.

As detailed in Table 5, significant variation between the monkeys of different origin was reported for the grading of lymphoplasmacytic infiltration in the two gastric regions and the colon ($p \leq 0.001$). A sparse gastric mucosal infiltration was present in Mauritian monkeys, whereas a moderate to marked diffuse infiltration of the antral mucosa was noted in those from The Philippines, and a moderate to marked diffuse infiltration of the fundic and antral mucosa was recorded in those from Vietnam. In the colon, the grade of infiltration was higher in Vietnamese animals.

The stomach was the only organ to present a correlation between the grade of LCI and the clinical data. As presented in Fig. 2, there was a positive correlation between the mean total WBC count and the mean LCI grade in the antral part of the stomach for monkeys from all three sources. This correlation was statistically assumed from the following coefficient of determination $R^2 = 0.989$. For the Mauritian cynomolgus monkeys, the lower WBC count correlated with the lower infiltration in the three parts of the digestive tract. For the Philippines and Vietnamese monkeys, the higher WBC count correlated with the higher grading of infiltration.

Kidneys: Lymphoplasmacytic cells were present in the renal interstitium of 54/90 cynomolgus monkeys. The involvement of tubules was used as the criteria to differentiate interstitial nephritis (IN) from simple LCI. IN was defined by an inflammatory infiltrate with tubular involvement, illustrated by a disrupted basement

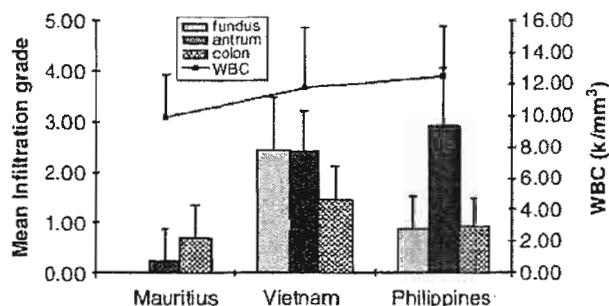


Fig. 2. Correlation between the mean lymphoplasmacytic infiltration grades in the digestive tract and the mean white blood cell count (WBC). A positive correlation between the antral grade of infiltration and the mean WBC count was considered to be statistically significant (coefficient of determination $R^2 = 0.989$).

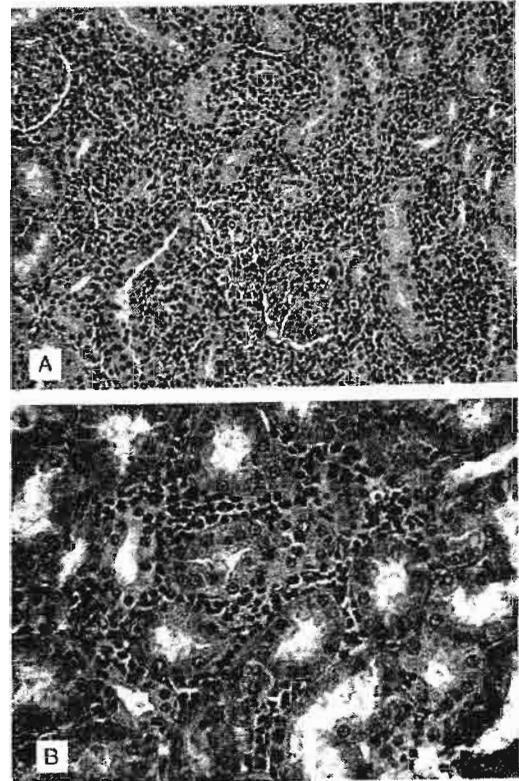


Fig. 3. Cynomolgus kidney: (A) interstitial nephritis, chronic, minimal, focal, with tubular involvement and (B) lymphoplasmacytic cell infiltration, with intact tubular basement membrane. HE stain, $\times 200$.

membrane, leucocytes exocytosis and tubular epithelial cell degeneration. The inflammatory infiltrate was mostly pleomorphic, with lymphocytes, plasmocytes, macrophages, rare granulocytes, often with red cell extravasation and karyorrhexis (Fig. 3A). The distribution was almost always focal or multifocal: only in one animal was this present as a diffuse unilateral IN with fibrosis. The cortex was more often affected than the medulla. In LCI there was an intact basement membrane and the infiltrate was composed of a majority of lymphocytes, present exclusively in the interstitium (Fig. 3B). A preferential distribution was noted in the medulla and in the adipose or connective tissue around the pelvis.

The lymphoplasmacytic infiltration grade was lower in Mauritian cynomolgus monkeys but did not attain statistical significance.

Heart: Focal inflammatory cell infiltrates were observed in the myocardium of 46/90 cynomolgus monkeys. They were often composed of predominance of lymphocytes, and occasionally as a mixture of lymphocytes, histiocytes and plasma cells. Mostly the lymphoid cells had infiltrated between the cardiac myofibres without disrupting them. These infiltrates were far more frequent in the ventricular myocardium than in the

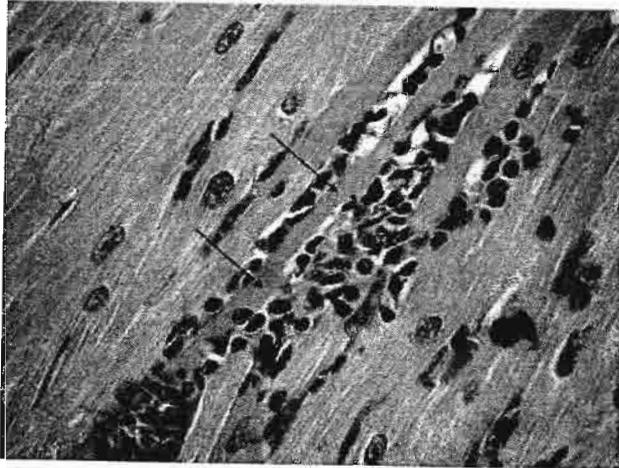


Fig. 4. Cynomolgus heart. Lymphoplasmacytic cell infiltration, minimal, focal, associated with macrophages and focal myofibre hyalinization (arrows). HE stain, $\times 400$.

atrial myocardium, but without any difference in the distribution on the left or right side of the organ. The subendocardial location was the most frequently affected, followed by the interstitial or subepicardial regions. In a few animals where the infiltrate was graded mild to moderate, there were associated degenerative myofibre changes that consisted of increased eosinophilia, a cellular retraction (circular profile), a loss of cross-striation and occasional macrophages (Fig. 4).

Liver: Inflammatory cell infiltrates were frequent in the liver (incidence 65/90), each hepatic location being infiltrated by different cells. Lymphoid cells were observed in the adventicia of the centrilobular vein (Fig. 5A) while mixed inflammatory cells were seen in the periportal area (Fig. 5B). Scattered lymphoplasmacytic cells were often found in the sinusal compartment without any hepatocyte involvement (Fig. 5C), and in the gallbladder submucosa.

Spleen: Variation in the cellularity of the white pulp was the main finding in the spleen. This consisted mostly of increased follicular cellularity (incidence 41/90) (Fig. 6A and B). There was less variation in the number of follicles or in the cellularity of the periarteriolar lymphocyte sheath. Several morphological appearances were seen in a same animal, indicating different stages of stimulation. Large-sized germinal centres showing mitosis were surrounded by a well-developed mantle zone of small lymphocytes, surrounded in turn by a large marginal zone with medium-sized lymphocytes. Tingible-body macrophages were often seen in these activated germinal centres. An accumulation of an acidophilic and amorphous substance accumulation was frequently observed in the centre of the follicle (centrofollicular hyalinosis), suggesting a protein content (possibly immunoglobulin) and a late-stage stimulation.

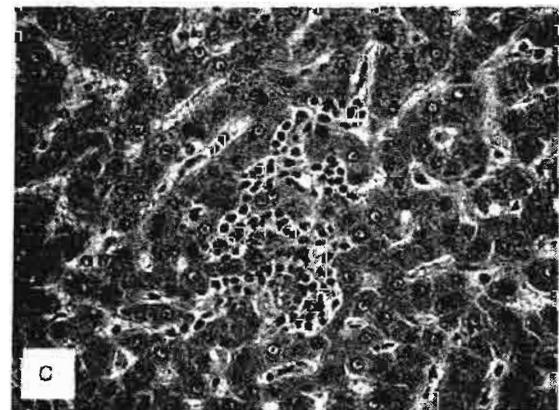
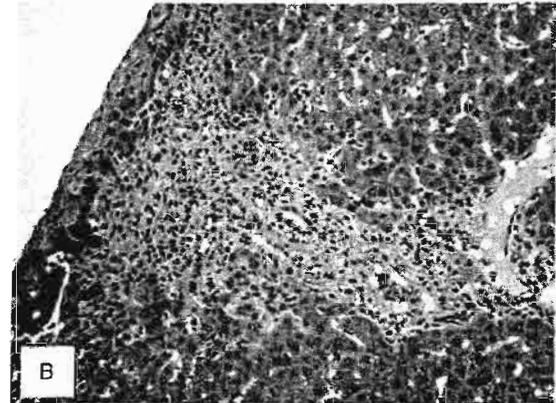
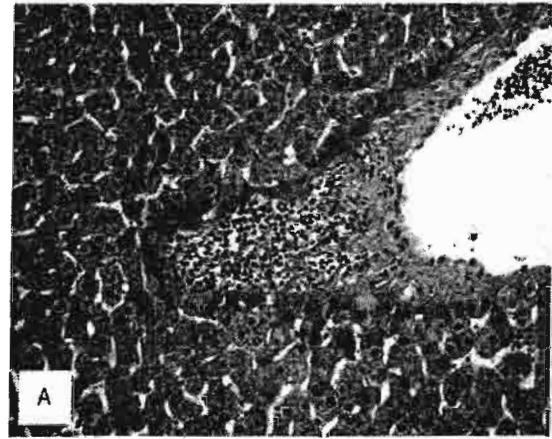


Fig. 5. Cynomolgus liver: (A) centrilobular lymphoid cell infiltration, minimal, focal. (B) periportal mixed inflammatory cell infiltration, chronic, minimal, focal and (C) sinusoidal lymphoplasmacytic cell infiltration, minimal, focal. HE stain, $\times 200$.

Lungs: Focal chronic interstitial pneumonia was diagnosed in 11/90 animals, but was always restricted to minimal to slight severity. Pneumonia consisted in a thickening of the alveolar wall by mixed inflammatory cells, type 2 pneumocytes hyperplasia or hypertrophy, with or without collagen deposition. These lesions were exclusively focal and were most frequent at the tip of pulmonary lobes.

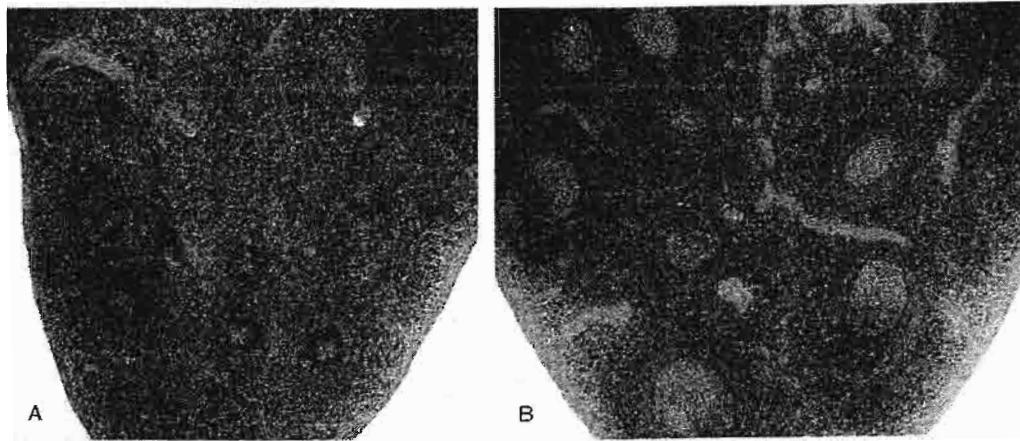


Fig. 6. Cynomolgus spleen: (A) non-increased cellularity of white pulp considered as baseline level and (B) moderately increased cellularity of follicles in white pulp. HE stain, $\times 40$.

Other microscopic findings

Digestive tract: Spiral-shaped bacteria were noted in the stomach of almost all cynomolgus monkeys. These microorganisms were observed at highest magnification in the superficial portions of the gastric epithelium, most frequently in the fundic region. The bacteria present in the fundic mucosa were often abundant, 4–10 μm long, and tightly coiled, features which are consistent with *Helicobacter heilmannii*-like organisms (Fig. 7). They were located in the gastric pits, superficial glands or even on the surface epithelium. The bacteria seen in the antral mucosa were often less numerous, shorter (3–5 μm long) and had a loose spiral- or comma-shape, features which are consistent with *Helicobacter pylori*-like organisms. These had a more superficial location in the mucosa. The *Helicobacter* organisms were observed with a very high incidence: all cynomolgus monkeys had at least a few bacteria in the fundic glands. However, no correlation was seen between their prevalence and the gastric inflammatory lesions described above.

Ciliated protozoan parasites consistent with *Balantidium coli* were frequently observed in the colonic or caecal lumen. The trophozoites were sometimes embedded in the most superficial crypts but they were not associated with any mucosal change (neither erosion, nor inflammation). These parasites seemed to be more frequent in the caecum than in the colon. The incidence in Mauritian cynomolgus monkeys (4/30) was significantly lower ($p \leq 0.001$) than in those from the Philippines (21/28) and Vietnam (21/32). Despite a similar incidence, the parasitic load was higher in The Philippines monkeys than in Vietnamese monkeys.

Pigmented macrophages were observed with a very high incidence in the lamina propria of the tip of duodenal, jejunal and ileal villusities, and in the superficial lamina propria of the colon and caecum. These macrophages were filled with a yellow to brown fine

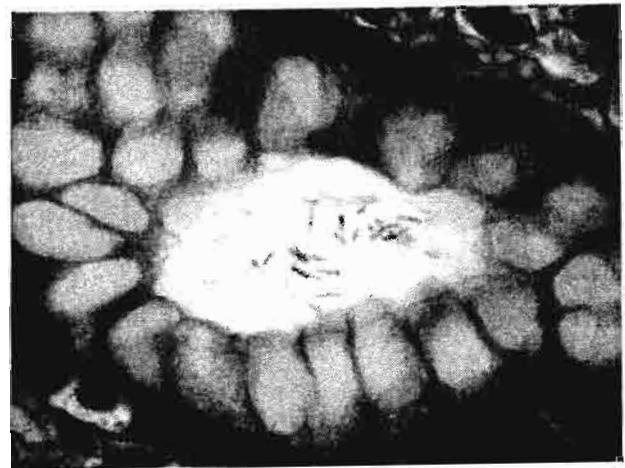


Fig. 7. Cynomolgus stomach. Section of a fundic pit containing 4–10 μm spiral bacteria consistent with *Helicobacter heilmannii*. HE stain, $\times 1000$.

granular intracytoplasmic pigment, which could suggest either bilirubin or lipofuscin/ceroid pigments. Frequent apoptotic bodies were interspersed between these macrophages, as well as some eosinophilic granulocytes (Fig. 8).

Nematodes resembling *Trichuris trichiura* were seen in the intestinal tract of three Vietnamese monkeys from the same study. One of the affected females had associated foreign body granulomata. Minimal multifocal mineralizations in the colonic mucosa were present in two females.

Kidneys: Cytoplasmic inclusions were occasionally seen in the urothelium of the pelvis, ureters, and vesical bladder. These inclusions appeared as brightly eosinophilic small (1–4 μm) intracytoplasmic granules, characterized by a superficial distribution throughout the urothelium (Fig. 9). The most superficial inclusions

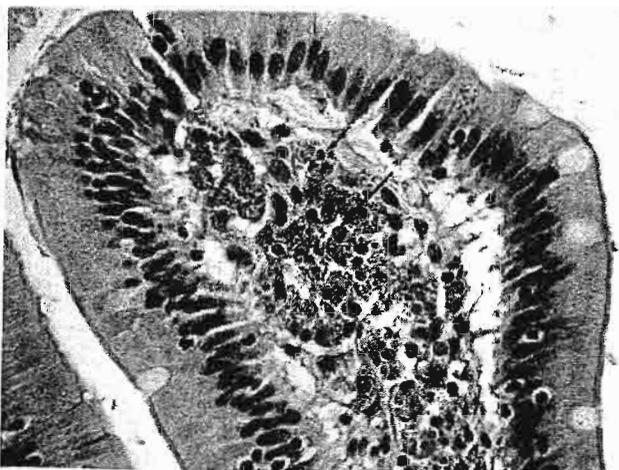


Fig. 8. Cynomolgus duodenum. Pigmented macrophages in the lamina propria of duodenal villi, associated with apoptotic bodies (short arrow) and occasional eosinophils (long arrow). HE stain, $\times 400$.

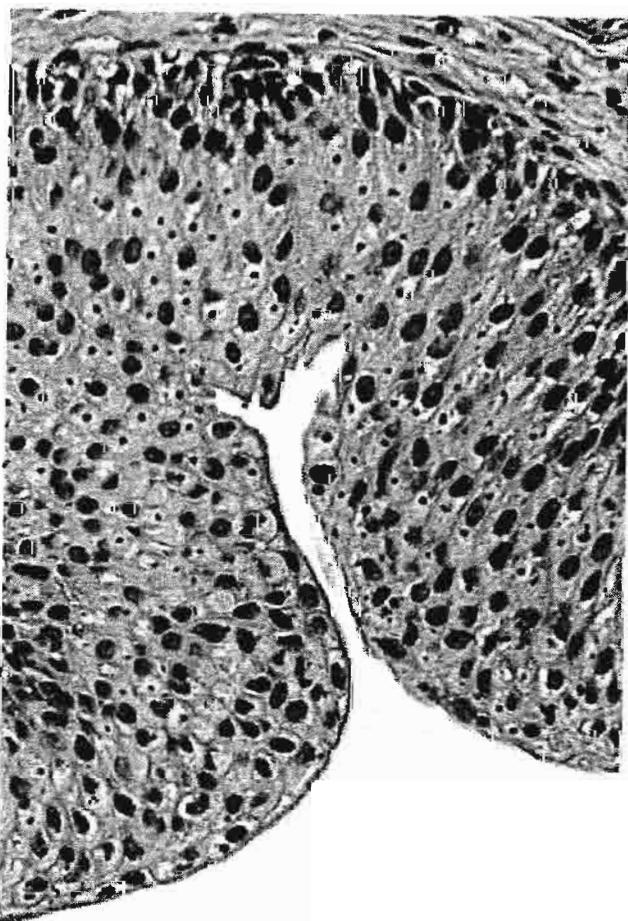


Fig. 9. Cynomolgus kidney. Eosinophilic intracytoplasmic inclusions, 1–4 μm , in the urothelial cells. HE stain $\times 400$.

frequently had a filamentous appearance. The inclusions were present in all cynomolgus monkeys from The Philippines (28/28) and almost all monkeys from Mauritius (27/30), whereas they were only occasionally seen in the Vietnamese monkeys (10/32). This finding was statistically significantly lower in cynomolgus monkeys from Vietnam ($p \leq 0.001$) than in the monkeys from the other two sources.

Glomerular degeneration was noted in five animals (three from Mauritius and two from The Philippines) and affected only a few glomeruli (always less than 10% of total glomeruli), not always bilaterally. These glomerular changes were characterized by the deposition of an amorphous eosinophilic substance, decreased cellularity and a generally expanded volume of the glomerulus, sometimes associated with periglomerular fibrosis. The distribution in the glomerulus was more frequently segmental than global (Fig. 10). There were no lesions in the other compartments. The eosinophilic substance was moderately positive with Masson's Trichrome stain, suggesting some collagenic content.

Immature/non-developed glomeruli were very frequent and were characterized by a small size, a basophilic appearance, many cuboidal cells composing the visceral surface of the Bowman epithelium, no expanded capillaries and no red blood cells. They were preferentially distributed in the superficial cortex and in the pelvic cortex.

Mineralization in the papilla was seen in 7/90 animals and were most frequent in the medulla and corticomedullary junction. Two other papillary changes had a similar distribution just under the urothelium and consisted of either oedema (incidence 5/90) or fibre



Fig. 10. Cynomolgus kidney. Glomerular degeneration, segmental, characterized by a slightly expanded volume, a decreased cellularity and the deposition of a pale eosinophilic, amorphous to slightly fibrillar substance. HE stain, $\times 400$.

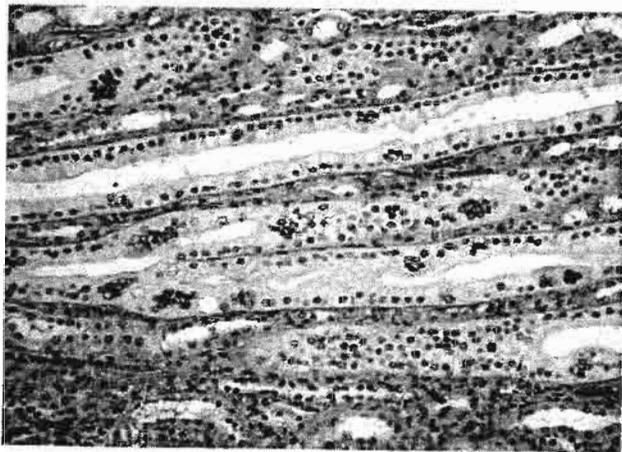


Fig. 11. Cynomolgus kidney. Multinucleated cells in the inner medullary collecting ducts of the papilla. HE stain, $\times 200$.

deposition (incidence 4/90). The oedema was represented by a myxoid appearance of the tip of the papilla, whereas the fibre deposition consisted in a pale, fibrillar, eosinophilic substance, with a low cellularity and slight Masson's Trichrome positivity.

Multinucleated cells were observed in the inner medullary collecting ducts of all cynomolgus monkeys (Fig. 11).

Heart: Scattered eosinophilic granulocyte infiltrates were occasionally seen in the epicardial adipose tissue. Small granulomata were noted in the epicardial adipose tissue of two animals. These granulomata were surrounded by inflammatory cells, predominantly eosinophils. Other findings such as minimal focal fibrosis, focal myocardial mucin deposition, ectopic epithelial structures (Fig. 12), blood-filled valvular cysts, etc. were present in only one animal each.

Liver: Centriobular glycogen retention was very frequent without any significant origin- or sex-relationship.

Binucleated hepatocytes, pigment in the gallbladder submucosa and tension lipidosis were also seen sporadically.

Spleen: A brown, finely granular pigment deposition (presumably haemosiderin) was commonly seen in the splenic macrophages. Other findings such as a lipidic granuloma, extramedullary foci of hemopoiesis and increased granulocytosis (myeloid hyperplasia) in the red pulp were seen occasionally. Granulocytosis was mainly composed of neutrophils and some eosinophils, and was more prominent near the capsule than in the centre of the parenchyma.

Lungs: Pigmented macrophages were frequently observed in a periarteriolar location and were filled with a black granular intracytoplasmic pigment which strongly suggested anthracosis. Foamy macrophages were pre-

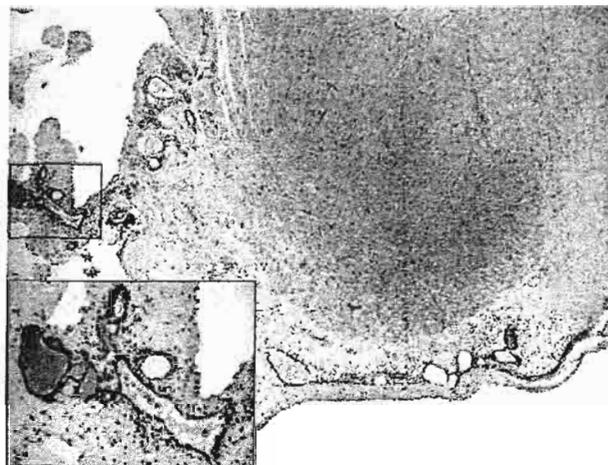


Fig. 12. Cynomolgus heart, interventricular septum. Ectopic epithelial structures filled with eosinophilic amorphous material and consistent with embryonic remnants. HE stain, $\times 40$ (inset $\times 200$).

sent with a multifocal intraalveolar distribution in four animals from the same study: the cytoplasm of these macrophages was distended with variably sized vacuoles. Focal to multifocal pleural fibrosis was seen in occasional monkeys.

Discussion

This study highlighted some differences between cynomolgus monkeys originating from different suppliers.

The terminal body weights and relative organ weight (except for the heart) were lowest in macaques from Vietnam, and this was considered to be partly correlated to their younger age. There were, however, some variations between the monkeys originating from Mauritius and The Philippines, although their ages were similar (30.3 and 30.7 months on average), suggesting some possible origin-related weight differences. The higher relative heart weight seen in Vietnamese monkeys could be related to their growing conditions in the breeding centre, such as the available exercise area.

The lower red blood cell count and compensatory higher mean corpuscular volume in monkeys from Vietnam could represent an origin-related variation. This difference is more convincing in view of the younger age of the Vietnamese monkeys, since the RBC count is reported to decrease with age, whereas Hb, MCV and MCH tend to increase (Terao, 2005). These parameters are easily influenced by frequent blood sampling but this was not the case in the studies using the Vietnamese monkeys. These values were similar to more extensive background data collected in

MDS from other studies. The higher eosinophil content in the blood of cynomolgus monkeys from The Philippines could be related to their higher intestinal parasitic load. The lymphocyte count was higher than the neutrophil count, especially for the males, as expected for cynomolgus monkeys less than five years old (Sugimoto et al., 1986). These proportions were reversed for the females, especially those from Mauritius. This could be explained by the earlier maturation of the females than males.

ALP values are reported to decrease with the age (Yoshida et al., 1986), so the lower ALP values in Vietnamese cynomolgus monkeys are likely to be age-related.

The main microscopic variations concerned LCIs in all the selected organs, mainly the digestive tract. The most important findings were observed in the stomach, where the lymphoplasmacytic infiltration grade varied significantly from one anatomic region to another, and according to the source of the monkeys.

The antral mucosa was generally more severely infiltrated than the fundic mucosa, especially in monkeys from The Philippines or Vietnam. Moreover, *Helicobacter* organisms were seen in the fundic mucosa of almost all cynomolgus monkeys, whereas they were less common in the antral mucosa and did not have the same morphologic features (Solnick and Schauer, 2001). These observations were similar to other reported results obtained in cynomolgus monkeys (Reindel et al., 1999) and baboons (Mackie and O'Rourke, 2003), which conclude that *H. pylori*-like organisms have a causative role in antral gastritis, whereas *H. heilmannii*-like organisms could be responsible for a relatively mild inflammation of the fundic mucosa. Moreover, the *H. pylori* strains isolated from five cynomolgus monkeys were shown to vary according to the geographical origin (Doi et al., 2005). Further investigation using immunohistochemistry to differentiate the organisms and a Warthin–Starry stain to help quantify the bacterial load, would clarify these findings.

As mentioned in Table 5, the global infiltration grade of the stomach was significantly lower ($p \leq 0.001$) in Mauritian cynomolgus monkeys than in those from other sources. A possible origin-related variation in susceptibility could explain why Mauritian cynomolgus monkeys are less frequently affected by a diffuse chronic gastritis. However, these differences could also be related to previous environmental factors in the breeding centres, such as nutrition or housing conditions. Outdoor-kept primates are believed to have a more important population of lymphocytes and plasma cells in their gastrointestinal tract lamina propria than indoor-housed primates (Lowenstine, 2003).

A higher incidence of *B. coli* was noted in cynomolgus monkeys from The Philippines and Vietnam, despite a similar antiparasitic treatment. This difference could be

explained by an origin-related variation in susceptibility. The higher parasitic load in the caecum might be only an artefact, the colonic mucosa being more efficiently cleaned than the caecum during necropsy. Nevertheless, *B. coli* is considered a non-pathogenic inhabitant of the caecum of immunocompetent monkeys (Purcell and Philipp, 2005), suggesting a greater prevalence in the caecum than in the colon.

The yellowish brown pigment found in macrophages of the superficial intestinal lamina propria was closely associated with apoptotic bodies. In a retrospective study (Ito et al., 1992), a Berlin blue stain was negative for this pigment, suggesting bile pigment although lipofuscin pigment must also be considered. From its very superficial location in the intestinal mucosa it could be assumed that this pigment represented either proteic or lipidic nutrient remnants.

Inclusion bodies in the transitional epithelium have been reported in rhesus macaques (Burek et al., 1972), and many other species, including cynomolgus macaques (Fussel and Roberts, 1979). These were considered by Burek (1972) not represent viral particles. Ultrastructural studies showed that these inclusions developed from compacting tonofilaments and were composed of keratin. A progression toward the epithelium was noted, inclusions becoming whorls of fingerprint-like structures in the most superficial cells.

The multifocal glomerular degeneration could account for an early-stage glomerulosclerosis, before the terminal glomerular collapse. Indeed a chronic membranous glomerulonephritis would have affected almost all glomeruli. For the same reason and because of Congo Red stain negativity, amyloidosis was ruled out. A collagenofibrotic glomerulonephropathy was recently described in one cynomolgus monkey (Adachi et al., 2005), showing a similar accumulation of a homogenous to slightly fibrillar substance in the mesangium. The glomerular lesions were diffuse and global, and accompanied by marked interstitial lesions such as lymphoplasmacytic infiltration, focally atrophic and dilated tubules with proteinaceous casts and peritubular fibrosis. Nevertheless, our glomerular findings could account for an earlier stage of this process. Lastly, a congenital glomerulosclerosis could be considered. This would be associated with ischaemic mechanisms involving arteriolar lesions and thus explaining the focal changes, but no vascular lesions were detected in our cases.

The non-developed glomeruli were frequently observed probably because of the immaturity of most monkeys. Dehydration could also be a factor in glomerular tuft shrinkage (Scott, 1992).

The ectopic epithelial structures observed in the interventricular septum of one cynomolgus monkey have been reported (Kaspereit et al., 2005) and are considered to be embryonic remnants of ectodermal origin.

Centrilobular glycogen retention is common in the liver of the cynomolgus monkey (Foster, 2005) and probably depends on the glycemic status of the animal and is influenced by alimentary or stress factors.

This study highlighted some variations between cynomolgus monkeys from different sources. The differences were particularly marked between the Mauritian cynomolgus monkeys and those originating from Southeast Asia (The Philippines and Vietnam): when compared with the Mauritian monkeys, the Asian cynomolgus monkeys had a significantly higher LCI grade in the examined organs, which correlated with an increased WBC count. Either the isolated geographical location of Mauritius and/or the variation in breeding centers' environmental factors may account for these variations. Microscopic examination of other organs would be useful to confirm this.

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Old World Nonhuman Primate Models of Type 2 Diabetes Mellitus

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Abstract

Type 2 diabetes mellitus is a major health problem of increasing incidence. To better study the pathogenesis and potential therapeutic agents for this disease, appropriate animal models are needed. Old World nonhuman primates (NHPs) are a useful animal model of type 2 diabetes; like humans, the disease is most common in older, obese animals. Before developing overt diabetes, NHPs have a period of obesity-associated insulin resistance that is initially met with compensatory insulin secretion. When either a relative or absolute deficiency in pancreatic insulin production occurs, fasting glucose concentrations begin to increase and diabetic signs become apparent. Pathological changes in pancreatic islets are also similar to those seen in human diabetics. Initially there is hyperplasia of the islets with abundant insulin production typically followed by replacement of islets with islet-associated amyloid. Diabetic NHPs have detrimental changes in plasma lipid and lipoprotein concentrations, lipoprotein composition, and glycation, which may contribute to progression of atherosclerosis. As both the prediabetic condition (similar to metabolic syndrome in humans) and overt diabetes become better defined in monkeys, their use in pharmacological studies is increasing. Likely due to their genetic similarity to humans and the similar characteristics of the disease in NHPs, NHPs have been used to study recently developed agonists of the peroxisome proliferators-activated receptors. Importantly, agonists of the different receptor subclasses elicit similar responses in both humans and NHPs. Thus, Old World NHPs are a valuable animal model of type 2 diabetes to study disease progression, associated risk factors, and potential new treatments.

Key Words: amyloid; diabetes; glucose; hormones; insulin; insulin resistance; monkeys; pancreas; obesity

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Introduction

In contrast to many chronic diseases, the prevalence of diabetes mellitus has increased substantially. Currently in the United States, roughly 21% of the population has diabetes. The increased prevalence of diabetes is primarily due to an increase in the type 2 diabetes mellitus (T2DM¹) population, who account for 90 to 95% of diabetic cases (American Diabetes Association, 2005). T2DM is associated with obesity in 85% of patients (Weir and Leahy 1994). In 2001, the prevalence of obesity was 20.9%, an increase of 5.6% from 2000 (Mokdad et al. 2003). Numerous complications are associated with diabetes, including both microvascular disease (e.g., retinopathy, neuropathy, and nephropathy) and macrovascular disease (e.g., coronary heart disease and stroke) (Bierman 1992).

Prediabetic conditions are also associated with increased health risks. For example, about half of the obese population has metabolic syndrome, classified by additional cardiovascular risk factors including increased blood pressure, increased plasma triglyceride concentrations, decreased high-density lipoprotein cholesterol (HDL¹) concentrations, and insulin resistance (Park et al. 2003). Insulin resistance is a key feature of obesity and T2DM; it may represent the earliest metabolic abnormality in the transition from normal to impaired glucose tolerance preceding the development of T2DM (DeFronzo 1997). As insulin resistance worsens, increasing amounts of insulin are needed to move glucose into target tissues, resulting in compensatory hyperinsulinemia.

Subclinical inflammation, which is associated with obesity, is thought to play an important role in both diabetes and cardiovascular disease. Adipose tissue is an important source of circulating inflammatory cytokines, representing a likely link between obesity and subsequent disease risk (Hotta et al. 2001; Kern et al. 2003). However, the timing of the onset of inflammation and its role in diabetes and cardiovascular disease are uncertain. In addition, it is unclear why pancreatic insulin production is sufficient in some obese humans or animals with insulin resistance, but not in

¹Abbreviations used in this article: AUC, area under the curve; DHEA-SO₄, dihydroepiandrosterone sulfate; HDL, high-density lipoprotein; HDLC, high-density lipoprotein cholesterol; HI, hyperinsulinemic; IAPP, islet amyloid polypeptide; IFG, impaired fasting glucose; IGT, impaired glucose tolerant; IVGTT, intravenous glucose tolerance test; LDL, low-density lipoprotein; NHP, nonhuman primate; PPAR, proliferator-activated receptor; T2DM, type 2 diabetes mellitus.

others. The answer is likely multifactorial, with interactions among environment, hereditary traits, and age.

T2DM develops naturally in several species of Old World nonhuman primates (NHPs¹), and they exhibit similar clinical features and pathological changes in the pancreas as those observed in humans. Additionally, similar risk factors for T2DM have been identified in NHPs and humans. Obesity, as mentioned before, may aggravate or precipitate diabetes by increasing insulin resistance, which in turn increases insulin requirement in tissues (Weyer et al. 1999). Aging itself is associated with increased insulin resistance, specifically with a decrease in acute insulin responses to glucose (Ramsey et al. 2000). In females, pregnancy, menopause, and sex hormone treatments can alter insulin resistance and may affect risk of T2DM (Bruns and Kemnitz 2004; Kavanagh et al. 2005; Kemnitz et al. 1988; Wagner et al. 2001, 1992). Furthermore, diet, housing conditions, and stress may affect insulin resistance and T2DM. As NHP models of metabolic syndrome and T2DM become better established, preclinical studies are being performed to test interventions of potential therapeutic importance.

Overview of Diabetes in Old World NHPs

Diabetes has been reported in a number of Old World NHPs, including many macaque species (e.g., cynomolgus, rhesus, bonnet, Formosan rock, pig-tailed, and celebes ma-

caques), African green monkeys, and baboons (Bodkin 2000; Clarkson et al. 1985; Cromeens and Stephens 1985; de Koning et al. 1993; Hansen and Bodkin 1986; Howard 1986; O'Brien et al. 1996; Ohagi et al. 1991; Tigno et al. 2004; Wagner et al. 1996a,b; Yasuda et al. 1988). As in humans, most NHPs with diabetes have T2DM (Wagner et al. 1996b, 2001). Similar to humans, T2DM is associated with increasing age and body weight in rhesus (Hamilton and Ciaccia 1978; Hotta et al. 2001), in cynomolgus monkeys (Wagner et al. 1996b), and in baboons (Banks et al. 2003; Cai et al. 2004; Stokes 1986). The disease initially is associated with normal glucose tolerance and insulin resistance with compensatory hyperinsulinemia, followed by continued deterioration of carbohydrate metabolism (Figure 1) (Hansen and Bodkin 1986). Cynomolgus (Wagner et al. 1996b, 2001) and rhesus monkeys (Bodkin 2000; Bodkin et al. 1993; Hansen and Bodkin 1986; Tigno et al. 2004) are insulin resistant and hyperinsulinemic for some time before development of overt diabetes.

As the disease progresses, NHPs develop impaired glucose tolerance with a slight elevation in fasting glucose before becoming overtly hyperglycemic due to a relative or absolute decrease in pancreatic insulin secretion. As discussed below, pancreatic exhaustion may eventually occur as normal islet architecture is replaced with islet-associated amyloid. Unlike the more rapid clinical presentation typical of type 1 diabetes, in T2DM, glucose and triglyceride concentrations can be elevated for several years before requiring intervention. However, with continued insulin resistance

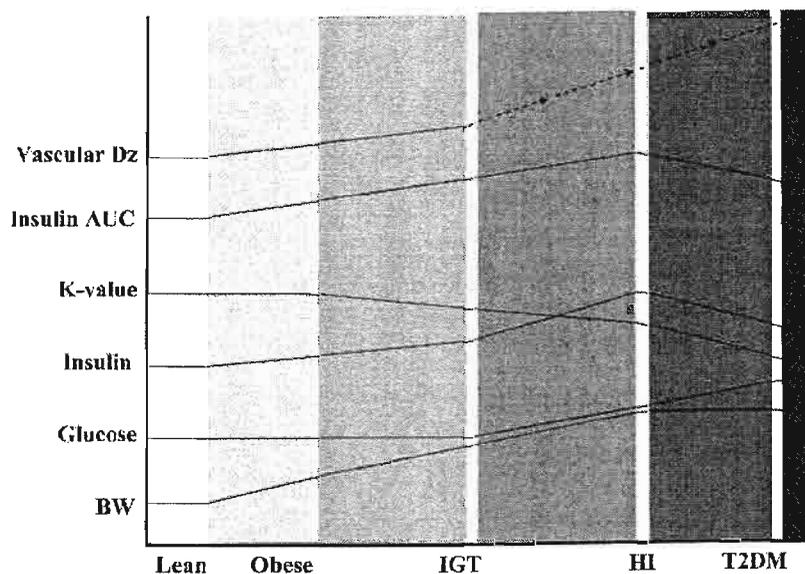


Figure 1 Theoretical progression from lean to obese with impaired glucose tolerance (IGT), hyperinsulinemia (HI), and type 2 diabetes mellitus (T2DM) in cynomolgus monkeys. Variables include body weight (BW), fasting glucose, insulin, and indices of glucose tolerance from intravenous glucose tolerance test (including glucose disappearance [K-value] and insulin area under the curve [insulin AUC]), and the proposed association with cardiovascular disease (Vascular Dz). Adapted from Bodkin NL. 2000. The rhesus monkey (*Macaca mulatta*): A unique and valuable model for the study of spontaneous diabetes mellitus and associated conditions. In: Sima AF, Shafir E. eds. *Animal Models in Diabetes: A Primer*. Singapore: Taylor & Francis, Inc. p 309-325.

and further declining pancreatic reserves, a sharp increase in glucose generally prompts treatment to prevent ketosis and acidosis (Wagner et al. 1996b). Caloric restriction can be a successful treatment for several years in some NHPs (Hansen et al. 1999; Wagner et al. 1996a), most likely due to improved insulin sensitivity (Bodkin et al. 2003; Cefalu et al. 2004; Gresl et al. 2001).

Clinical Parameters Associated with Early Changes in Progression to T2DM

Fasting glucose is most often measured as an indicator of T2DM risk. Overt diabetes is classified as a fasting glucose level 126 mg/dL, while an abnormal 2-hr postprandial glucose concentration (measured via oral glucose tolerance test) of 140 mg/dL is diagnostic of impaired glucose tolerance in humans (American Diabetes Association 2005). In monkeys, fasting glucose concentrations are about 20 to 30 mg/dL lower than humans' (average human basal glucose 85 mg/dL; Merimee and Tyson 1977); thus a fasting glucose in the impaired fasting glucose range of 100 to 126 mg/dL is more likely to indicate overt diabetes (Table 1). However, numerous clinical changes occur long before fasting glucose is elevated. Longitudinal measurement of plasma insulin concentrations is important because elevations occur long before changes in fasting glucose. Even more important than fasting glucose or insulin concentrations are stimulated glucose and insulin responses following a glucose challenge. In humans, the acute insulin response to a glucose challenge is impaired years before the development of glu-

cose intolerance and diabetes (DeFronzo 1997; Weyer et al. 1999).

Hyperinsulinemic euglycemic clamp studies are considered the gold standard for assessing insulin resistance, and their use in NHPs has been validated in healthy and diabetic rhesus monkeys (Bodkin et al. 1989). In these studies, supraphysiological concentrations of insulin are maintained while the ability of tissues, mainly skeletal muscle, to maximally dispose of glucose in response to insulin is assessed. Reductions in peripheral tissue sensitivity to insulin is seen early in the pathogenesis of T2DM, corresponding with β -cell hypersecretion of insulin in response to glucose (Hansen and Bodkin 1990; Ortmeier et al. 1993a). Clinically, tissue resistance to insulin is then compensated by hyperinsulinemia, until pancreatic dysfunction and insulin resistance combine to induce impaired glucose tolerance and, finally, T2DM (Weyer et al. 1999). The initial resistance is the result of post-insulin receptor activation signaling abnormalities. In rhesus monkeys, muscle cells show defective basal and insulin-mediated activation of glycogen synthase in peripheral tissues (muscle and adipose) in the earliest stages of insulin resistance. Alterations in liver metabolism were not detected, which may reflect a later development in the progression to T2DM (Ortmeier and Bodkin 1998; Ortmeier et al. 1993a,b).

Our group used intravenous glucose tolerance tests (IVGTTs¹) (Wagner et al. 1996a,b) to characterize different stages of disease progression in a group of nondiabetic cynomolgus monkeys (Figure 2). All monkeys were fed a high carbohydrate and low cholesterol (0.026 mg/calorie) experimental diet that contained 18% protein, 22% fat, and 60%

Table 1 Clinical parameters in nondiabetic cynomolgus monkeys that were classified as control, hyperinsulinemic (HI), impaired glucose tolerant (IGT) or both (HI+IGT) based on intravenous glucose tests

Group N = Male/Female	Control ^a N = 7/5	HI ^b N = 5/3	IGT ^c N = 3/7	HI + IGT ^d N = 4/1	<i>p</i> Value	Adjusted <i>p</i> Value (age + body weight)	Post-Hoc <i>p</i> < 0.05
Age (yr)	14.2 ± 2.5	12.0 ± 2.5	19.9 ± 1.8	18.4 ± 2.6	0.10	0.05	ns*
Body Weight (kg)	6.0 ± 0.9	7.2 ± 0.9	5.4 ± 1.0	10.3 ± 1.6	0.04	0.02	d ≠ c
Male/Female	8.1 ± 0.8/3.2 ± 0.2	9.0 ± 0.2/4.1 ± 0.5	9.9 ± 0.9/3.4 ± 0.2	11.1 ± 1.7/6.9			
Glucose (mg/dL)	55.2 ± 2.4	69.0 ± 4.5	62.0 ± 4.0	89.2 ± 10.3	<0.001	<0.001	d ≠ a, c d ≠ a, b, c
Glucose AUC*	8484 ± 349	10073 ± 747	13901 ± 767	17420 ± 1590	<0.001	<0.001	c ≠ a, b d ≠ a, b c ≠ a, b d ≠ a, c
Glucose K-value	5.2 ± 0.3	4.5 ± 0.2	2.1 ± 0.2	1.5 ± 0.2	<0.001	<0.001	c ≠ a, b d ≠ a, c
Insulin (μ IU/mL)	12.8 ± 2.2	56.5 ± 10.4	15.1 ± 1.6	62.7 ± 13.5	<0.001	<0.001	c ≠ b b ≠ a
Insulin AUC	7283 ± 1178	10349 ± 1052	4955 ± 1000	9127 ± 1429	0.02	0.11	ns*
Leptin (ng/mL)	7.2 ± 2.4	6.5 ± 1.4	10.2 ± 2.3	31.1 ± 7.0	0.003	0.03	d ≠ a, b
Cholesterol (mg/dL)	136 ± 6.9	141 ± 12.8	151 ± 29.6	130 ± 20.1	0.92	0.86	ns*
HDLC* (mg/dL)	67 ± 3.9	72 ± 5.4	51 ± 4.2	49 ± 9.1	0.01	0.06	ns*
Triglycerides (mg/dL)	86 ± 12.1	68 ± 6.1	73 ± 12.9	220 ± 66.2	0.02	0.02	d ≠ a, b, c

*AUC, area under the curve; HDLC, high-density lipoprotein cholesterol; ns, not significant.

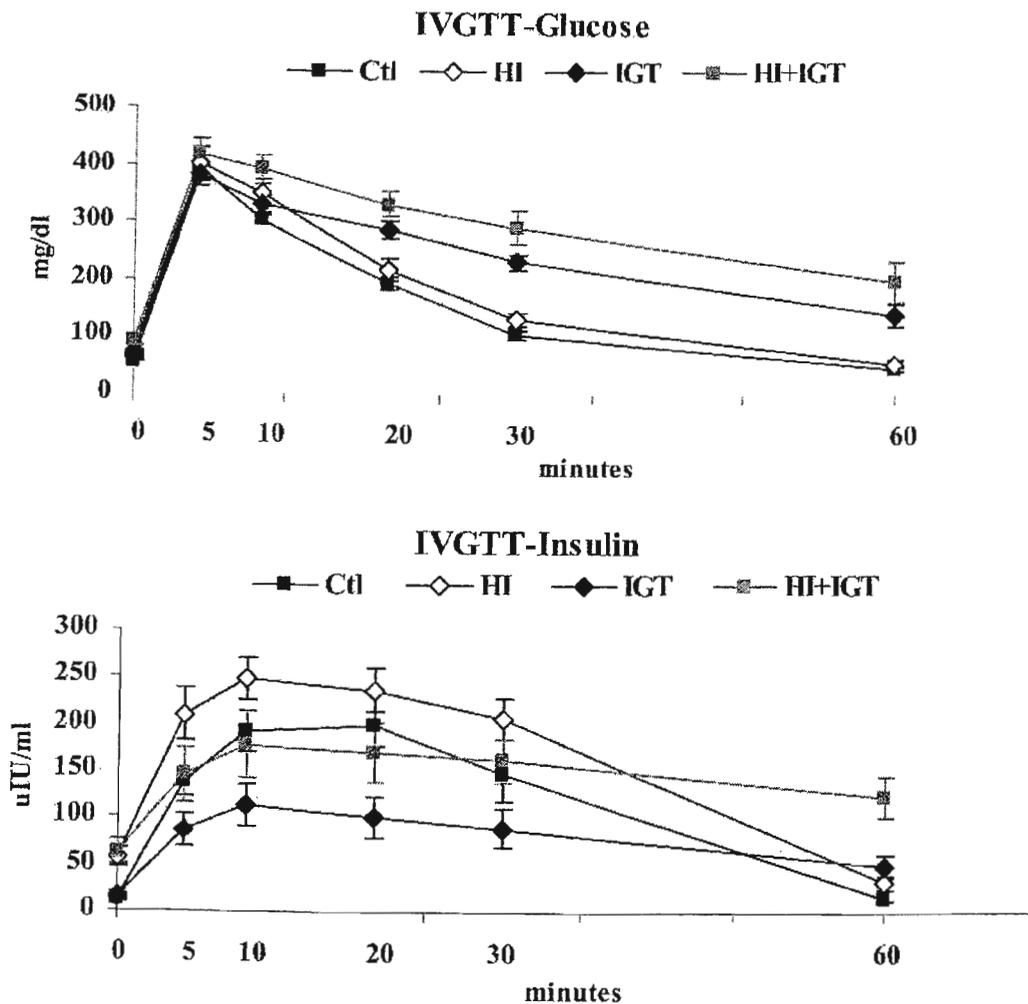


Figure 2 Glucose (top) and insulin (bottom) responses during intravenous glucose tolerance tests (IVGTTs) in cynomolgus monkeys classified as control (Ctl) or those with fasting hyperinsulinemia (HI), impaired glucose intolerance (IGT), or both HI and IGT (HI+IGT).

carbohydrate with increased fructose (20 g fructose/100 g dry weight). Most monkeys had relatively normal fasting glucose concentrations; however, their plasma glucose and insulin responses to the glucose challenge were very different. Based on the response to the IVGTT (Figure 2), we classified monkeys as either control ($n = 12$), hyperinsulinemic (HI¹) ($n = 8$), impaired glucose tolerant (IGT¹) ($n = 10$), or both IGT and HI ($N = 5$). Hyperinsulinemic was defined as a fasting insulin $30 \mu\text{ IU/mL}$, and impaired glucose tolerant was defined as 1-hr post-IVGTT glucose $>80 \text{ mg/dL}$.

Results of the IVGTT studies are presented in Table 1. Fasting insulin concentrations were slightly lower than previously reported (Wagner 2001) because newer assays used in this report (Merckodia, Uppsala, Sweden) do not cross-react with proinsulin. The lean control monkeys had normal fasting glucose concentrations of 55 mg/dL and insulin concentrations of $12.8 \mu\text{ IU/mL}$. By definition, HI monkeys

had increased fasting insulin concentrations ($57 \mu\text{ IU/mL}$; $p < 0.001$). Despite being slightly heavier, their glucose disappearance curves were similar to those in controls due to the compensatory increase in insulin secretion. IGT monkeys had only slightly higher fasting glucose concentrations compared with controls, but their stimulated glucose concentrations did not return to normal within 1 hr. This characteristic was due to slower glucose clearance (lower K-value), thus glucose areas under the curve (AUC¹) were increased. Those with both HI and IGT had significantly increased fasting glucose and insulin concentrations, and increased glucose and insulin AUCs. HI+IGT monkeys were also obese, with average body weights 40% greater than other monkeys. As expected with increased body weight, plasma leptin concentrations were also greater in this group, which has been shown to be associated with obesity in rhesus monkeys (Ramsey et al. 2000) and development of the metabolic syndrome in humans (Franks et al.

2005). Furthermore, all monkeys described in Table 1 had increased plasma triglyceride and lower HDLC concentrations compared with the chow-fed control monkeys (described below) due to the experimental diet, with the HI+IGT group having the highest triglyceride levels and the lowest HDLC. One could consider monkeys in the IGT and the HI+IGT groups as “prediabetic,” because within 1 yr after this classification, one of 10 monkeys in the IGT group and three of five monkeys in the HI+IGT group became diabetic.

Clinical Parameters Associated with T2DM in Monkeys

We compared spontaneous T2DM monkeys ($n = 25$) with recent adult male imports ($n = 24$). All monkeys were fed commercial monkey chow (Purina Lab Diet #5038). As shown in Table 2, the T2DM monkeys are older and have greater body weights (thus p values are shown both unadjusted and adjusted for age and body weight). Once monkeys become diabetic, they develop dyslipidemia and glucosuria, lose weight, and eventually become ketotic without treatment (Wagner et al. 1996a,b). Although a variety of oral agents or caloric restriction may effectively control T2DM in many monkeys, we have found it useful to treat all diabetic animals with insulin to standardize experimental conditions for intervention studies. Thus all of the diabetic monkeys described in Table 2 were treated with insulin twice daily (70/30 Novolin, Novo Nordisk Pharmaceuticals, Princeton, NJ). The afternoon before blood sampling, monkeys were given regular insulin (Novolin R, Novo Nordisk Pharmaceuticals) so that exogenous insulin was no longer present the following morning. Thus their fasting glucose concentrations were significantly increased (Table 2). The increase in glucose concentrations along with increased insulin concentrations are indicative of insulin resistance.

Unlike most adipocytokines, which are generally associated with increased inflammation, adiponectin is associated with decreased inflammation, improved insulin sensitivity, and improved vascular reactivity (Fernandez-Real et al. 2004; Tan et al. 2004). Thus, as expected, adiponectin concentrations are significantly lower in diabetic monkeys. Similar findings were reported in rhesus monkeys; as they progressed from insulin resistance to T2DM, their adiponectin concentrations decreased in parallel with reductions in insulin sensitivity (Hotta et al. 2001). Leptin concentrations are often increased in T2DM, correlating significantly with body weight (Figure 3). Interestingly, leptin concentrations increased in these rhesus monkeys as they became more insulin resistant and obese, but decreased as they became diabetic and lost body fat, similar to our experience (Tables 1 and 2) (Hotta et al. 2001).

Diabetic monkeys also have increases in plasma triglyceride and total cholesterol concentrations, similar to those seen in diabetic humans (Table 2). In monkeys, this characteristic is most often due to elevated triglyceride-rich particles, such as very low density lipoprotein (VLDL) particles, with relatively little change in low-density lipoprotein (LDL¹) cholesterol and HDLC concentrations (Bagdade et al. 1995; Wagner et al. 2001). Elevated triglycerides in relatively insulinopenic monkeys are most likely due to impaired lipoprotein lipase activity, which is insulin dependent and plays a major role in catabolism of triglyceride-rich lipoproteins. Also similar to human diabetics, cholesteryl ester transfer is increased and correlates with glycemic control (Bagdade et al. 1995). This characteristic causes cholesteryl ester enrichment of some particles (e.g., LDL) and results in more atherogenic particles, thus possibly contributing to increased atherosclerosis in diabetic monkeys. Additionally, HDLC is generally lower in diabetics, but this was not apparent due to the inclusion of females in the diabetic group.

Glucose control can be more accurately assessed in

Table 2 Clinical parameters in nondiabetic control and diabetic cynomolgus monkeys

N = Male/Female	Control N = 24/0	Diabetic N = 17/8	p Value	Adjusted p Value (age + body weight)
Age (yr)	14.92 ± 0.53	19.16 ± 0.93	0.0003	0.002 (body weight)
Body Weight (kg)	4.8 ± 0.12	8.15 ± 0.65	<0.0001	<0.0001 (age)
Male/Female		9.84 ± 0.51/4.58 ± 0.73		
Glucose (mg/dL)	57.46 ± 1.63	260.12 ± 21.74	<0.0001	<0.0001
Insulin (μ IU/mL)	11.42 ± 0.93	90.59 ± 18.5	<0.0001	0.031
Fructosamine (μ mol/L)	184.51 ± 8.59	434.14 ± 33.43	<0.0001	<0.0001
FFA* (mEq/L)	0.75 ± 0.04	1.5 ± 0.14	<0.0001	<0.0001
Leptin (ng/mL)	0.64 ± 0.24	15.53 ± 2.31	<0.0001	<0.0001
Adiponectin (pg/mL)	24.8 ± 3.77	11.59 ± 3.53	0.001	0.53
Cholesterol (mg/dL)	107 ± 3.8	164 ± 15	<0.0001	<0.0001
HDLC* (mg/dL)	40 ± 1.6	42 ± 2.9	0.72	0.008
Triglycerides (mg/dL)	31 ± 3.8	353 ± 48	<0.0001	<0.0001

*FFA, free fatty acid; HDLC, high-density lipoprotein cholesterol.

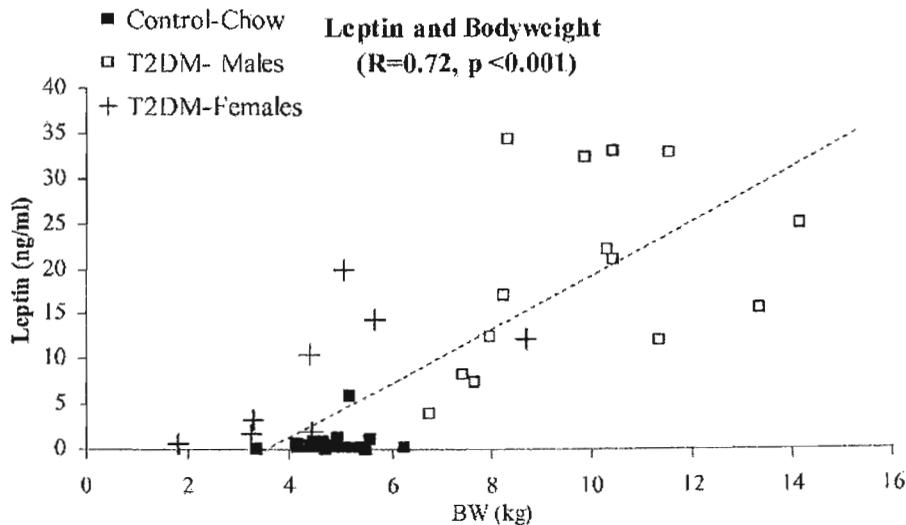


Figure 3 Relationship between plasma leptin concentrations and body weight (BW) in male control monkeys and male and female monkeys with type 2 diabetes mellitus (T2DM). All monkeys were fed standard chow diets. All T2DM monkeys were treated twice daily with insulin (70/30 Novolin, Novo Nordisk Pharmaceuticals, Princeton, NJ).

NHP models by measuring glycated proteins, as opposed to fasting glucose (Cefalu et al. 1993; Wagner et al. 1996a), because these proteins increase in conjunction with elevations of both fasting and postprandial glucose levels. As in humans, fructosamine concentrations in T2DM monkeys (an indication of albumin glycation) are increased (Table 2). Besides the typically measured increases in albumin (fructosamine) or hemoglobin glycation (GHb or HbA1c), hyperglycemia will result in increased glycation of lipoproteins, such as LDL particles. T2DM monkeys had similar increases in glycated hemoglobin and in LDL glycation (Figure 4); furthermore, there was a significant correlation between glycated hemoglobin and glycated LDL ($r = 0.59, p < 0.05$) (Wagner et al. 1996a). The glycation of LDL decreases its recognition by the LDL receptor, leading to increased uptake by macrophages (e.g., in the artery wall), thus exacerbating atherosclerosis. These changes in

lipoprotein composition and glycation may explain some of the increased risk of atherosclerosis in diabetics, which is present even when plasma cholesterol concentrations are relatively normal (Bierman 1992). In a chemically induced model of diabetes in monkeys, elevated glucose concentrations resulted in glycation of LDL particles. This effect promoted LDL interaction with matrix proteins of the artery wall, likely resulting in increased oxidative damage and increased atherosclerosis (Edwards et al. 1999; Litwak et al. 1998; Pennathur et al. 2001).

Islet Amyloidosis Associated with T2DM

Both in humans (Johnson et al. 1992; O'Brien et al. 1993) and in nonhuman primates (de Koning et al. 1993; Howard 1974; O'Brien et al. 1996; Wagner et al. 1996b) with T2DM,

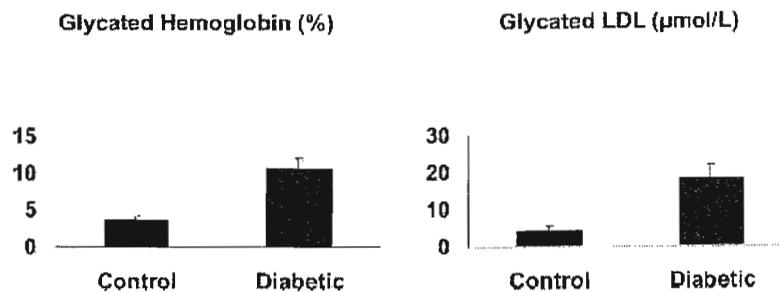


Figure 4 Glycated hemoglobin (%) and glycated low-density lipoprotein (LDL, $\mu\text{mol/L}$) values for nondiabetic and cynomolgus monkeys with type 2 diabetes mellitus. Modified from Wagner JD, Carlson CS, O'Brien TD, Anthony MS, Bullock BC, Cefalu WT, 1996. Diabetes mellitus and islet amyloidosis in cynomolgus monkey. *Lab Anim Sci* 46:1254-1262.

the major effect on the pancreas is islet amyloidosis. Islet amyloid is found in about 90% of humans with T2DM; as with monkeys, the degree of islet mass replaced by amyloid appears to correlate with increasing insulin requirements (Kahn et al. 1999). Amyloid is toxic to β -cells, inciting apoptosis (Lorenzo et al. 1994), and it may play a role in the reduced glucose tolerance of aging. However, age-associated amyloidosis may not be as important as changes in body composition. This possibility is evidenced by studies of caloric restriction in monkeys (Cefalu et al. 2004) and studies documenting lean elderly people as insulin sensitive as lean youths (Bryhni et al. 2003). Islet amyloidosis has been reported in T2DM *Macaca mulatta* (de Koning et al. 1993), *Macaca nigra* (Howard 1986), *Macaca nemestrina* (Ohagi et al. 1991), *Macaca fascicularis* (Cromeens and Stephens 1985; O'Brien et al. 1996), and baboons (Hubbard et al. 2002).

We reported that the amyloid is immunoreactive for islet amyloid polypeptide (IAPP¹) and is generally associated with a marked reduction in insulin-immunoreactive β -cells (O'Brien et al. 1996; Wagner et al. 1996b, 2001). IAPP has been reported in humans, cats, and monkeys (Hoppener et al. 2000). Rodents and many other species have differences in the amino acid sequence of one region of the protein (residues 20-29) that is most responsible for the formation of amyloid fibrils. Rodents transgenic for the human IAPP gene develop islet amyloid and have been useful in studying the importance of amyloid in the pathogenesis of T2DM (Kahn et al. 1999; Matveyenko and Butler 2006). We have also found islet amyloid deposition in nondiabetic monkeys. Little if any amyloid is found in young monkeys, whereas

increasing amounts are found in older, more insulin-resistant monkeys, although still less than in diabetic monkeys when matched for age (O'Brien et al. 1996; Wagner et al. 1996b). Thus in macaques, amyloid deposition often precedes overt diabetes (Hoppener et al. 2000; O'Brien et al. 1996; Wagner et al. 1996b). Defects in insulin secretion occur early in the pathogenesis of diabetes (DeFronzo 1997; Weyer et al. 1999), and the relationship between early deposition of amyloid and defects in insulin secretion may be an important feature in the progression of T2DM (Kahn et al. 1999).

Consistent with the islet cell abnormalities discussed above are the insulin-immunostained islet cells from an age-matched control and two T2DM monkeys shown in Figure 5 (Wagner et al. 2001). Pancreatic islets from diabetic monkeys (Figure 5, B and C) are larger and hyperplastic compared with nondiabetic age-matched controls (Figure 5A) (O'Brien et al. 1996). Islets from early T2DM cases have intense insulin immunostaining (Figure 5B), whereas islets from a monkey that had been diabetic for many years have less (Figure 5C). In addition, islets from monkeys with advanced T2DM are hypocellular, with abundant islet amyloidosis (Figure 5D). The islet amyloid was found to be immunoreactive for human IAPP, and the amino acid sequence was similar to that of humans (O'Brien et al. 1996).

Sex Hormones and Risk of T2DM

Estrogen and progestogens affect body weight and insulin resistance, thereby influencing the risk of both diabetes and cardiovascular disease (Wagner 2001). Pregnancy, for ex-

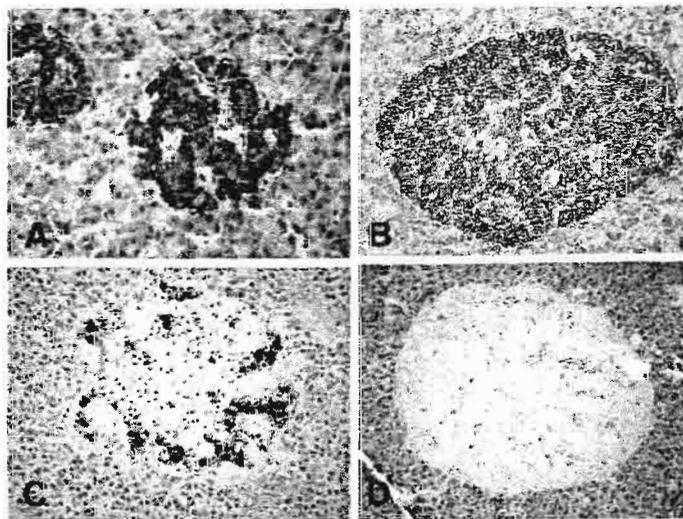


Figure 5 Photomicrographs of sections of pancreas immunostained for insulin from a nondiabetic monkey (A), and monkeys with early type 2 diabetes mellitus (T2DM) (B) and late T2DM (C). Abundant islet amyloid deposition (hematoxylin and eosin staining) is shown (D) from a T2DM monkey. Objective magnification for all, 20 \times . Modified and reprinted with permission from Toxicologic Pathology: Wagner JD, Cline JM, Shadoan MK, Bullock BC, Rankin SE, Cefalu WT. 2001. Naturally occurring and experimental diabetes in cynomolgus monkeys: A comparison of carbohydrate and lipid metabolism and islet pathology. *Toxicol Pathol* 29:142-148.

The importance of nonhuman primate models for the study of geriatric diseases and therapies

By Gerhard Weinbauer, PhD

Over the past centuries, life expectancy has been steadily increasing and shows no signs of slowing down. In many countries the percentage of the population 65 years of age or older will exceed 20% by 2020 and in the US, it is estimated that more than 13 million people will be age 85 or older by the year 2040 (Figure 1). Inevitably, the prevalence of aging-related diseases and deficiencies will gain further significance and the health status of the aged will have a great bearing on society and economy. Humans in developed parts of the world are living longer with diseases and dysfunctions becoming manifest well before the end of normal life expectancy.

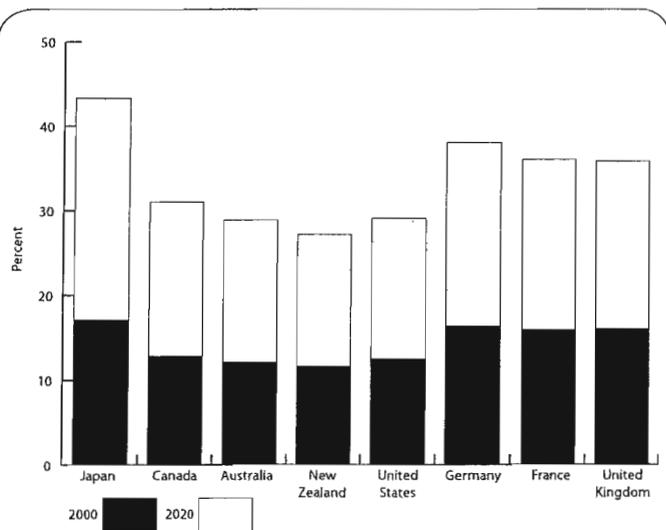
The cost of treating these individuals is increasing at least proportionally if not at an even greater pace. In the US, the annual health care cost for persons over the age of 85 has almost doubled in the past 20 years and there is widespread concern by most governments and international agencies that the cost currently being set aside to pay for these diseases will be inadequate.

The increasing and still increasing life span of humans is associated with an increased incidence of various pathologies and malignant diseases such as prostate diseases, ocular pathology, osteoporosis, diabetes, and Alzheimer's disease. This presents a significant challenge to the pharmaceutical industry for developing sufficient and appropriate medications for the elderly and aged population. The need for more relevant geriatric models for use in drug development is related to several issues. The first is closely tied to the demographics emerging in developed and developing countries over the past decade. In addition, there will be a need for new medicines that are safer and can be taken over long periods even if therapy is initiated late in an individual's life. For the successful development of new therapies for geriatric diseases, relevant animal models will be of vital importance.

The Relevance of Nonhuman Primate Models

The past decade has seen an improvement in the ability to create animal models of disease through genetic manipulations such as the construction of transgenic rodents carrying altered genes or in some cases human gene constructs. However, the aging process of rodents is often dissimilar to primates resulting in models that may not be useful in developing the most effective therapies for geriatric diseases. The inherent benefits derived from the development of nonhuman primate models are the relevance of disease to the aged human. The nonhuman primate is an appropriate model for the evaluation of a compound's effect on numerous bodily systems and functions. As a result, nonhuman primates are used for a variety of study types including subchronic and chronic toxicology, reproductive and developmental toxicity studies, safety pharmacology, and pharmacokinetic evaluations. In addition, these animals are

Figure 1. Percentage of Populations Age 65+ in 2000 and 2020



Source: United Nations

valuable models in the study of the absorption, distribution, metabolism, and excretion (ADME) of compounds. Due to the similarities to the human immune and central nervous systems, nonhuman primates provide unique opportunities to seek solutions to problems associated with cancer, infectious and geriatric diseases. Nonhuman primates are also valuable in the study of the specific effect of a compound on the reproductive, bone, and ocular systems.

Another key aspect of the value of the nonhuman primate is the increasing proportion of new pharmaceutical candidates that are biotechnology-derived protein medicines. In this context, nonhuman primates may constitute the relevant animal model. As gene therapies move from the research arena into the preclinical and clinical developmental phases, the use of nonhuman primate models with closer genetic identity to humans should optimize therapeutic effectiveness. Nonhuman primates are excellent models for studying age-related changes in anatomy, physiology, behavior, and mental function. This research has been especially helpful for understanding neurological phenomena like learning and memory deficits, visual impairment, neurochemical changes, and neuropathology.

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The establishment of breeding colonies on the island of Mauritius has had a significant impact on the availability of animals for these studies. Mauritian male and female cynomolgus monkeys are retained as retired breeders (typically more than 13 years old) from a breeding colony. A side benefit of the breeding colonies has been the opportunity to conduct research on the impact of aging that might otherwise have been unavailable. The use of Mauritian primates has resulted in a major improvement in the quality of the animals and a significant reduction in handling risks as there is no longer the danger of Herpes B infection. Mauritian cynomolgus monkeys are genetically devoid of herpes B virus and seem to have a lower degree of genetic variability

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Addressing Alzheimer's Disease

Alzheimer's disease (AD) in its advanced stage affects approximately 15 million people worldwide and studies show the potential of affecting 50 million by 2030. The

The importance of nonhuman primate models for the study of geriatric diseases and therapies

By Gerhard Weinbauer, PhD

Over the past centuries, life expectancy has been steadily increasing and shows no signs of slowing down. In many countries the percentage of the population 65 years of age or older will exceed 20% by 2020 and in the US, it is estimated that more than 13 million people will be age 85 or older by the year 2040 (Figure 1). Inevitably, the prevalence of aging-related diseases and deficiencies will gain further significance and the health status of the aged will have a great bearing on society and economy. Humans in developed parts of the world are living longer with diseases and dysfunctions becoming manifest well before the end of normal life expectancy.

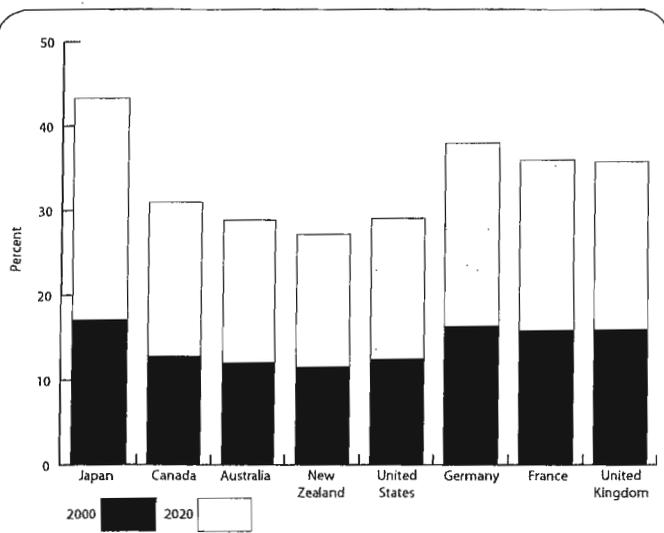
The cost of treating these individuals is increasing at least proportionally if not at an even greater pace. In the US, the annual health care cost for persons over the age of 85 has almost doubled in the past 20 years and there is widespread concern by most governments and international agencies that the cost currently being set aside to pay for these diseases will be inadequate.

The increasing and still increasing life span of humans is associated with an increased incidence of various pathologies and malignant diseases such as prostate diseases, ocular pathology, osteoporosis, diabetes, and Alzheimer's disease. This presents a significant challenge to the pharmaceutical industry for developing sufficient and appropriate medications for the elderly and aged population. The need for more relevant geriatric models for use in drug development is related to several issues. The first is closely tied to the demographics emerging in developed and developing countries over the past decade. In addition, there will be a need for new medicines that are safer and can be taken over long periods even if therapy is initiated late in an individual's life. For the successful development of new therapies for geriatric diseases, relevant animal models will be of vital importance.

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Addressing Alzheimer's Disease

Alzheimer's disease (AD) in its advanced stage affects approximately 15 million people worldwide and studies show the potential of affecting 50 million by 2030. The

Table 2. Comparison of selected clinical pathology parameters in young (< 6 years) and old (> 20 years) female cynomolgus monkeys

Parameter	Non-Geriatric Animals			Geriatric Animals		
	No. of Animals	Mean	Range	No. of Animals	Mean	Range
Triglycerides (mmol/L)	116	0.59	0.28-1.14	29	1.52	0.63-5.56
Cholesterin (mmol/L)	156	3.06	1.47-4.57	29	2.73	2.01-3.74
HDL (mmol/L)	83	1.03	0.58-1.73	29	1.14	0.60-1.88
LDL (mmol/L)	83	1.68	0.71-2.97	29	1.21	0.17-2.42
Glucose (mmol/L)	156	3.58	1.62-5.84	29	5.40	3.61-7.99

main histological features of AD are extracellular peptide deposits termed β -amyloid (or senile) plaques, vascular β -amyloid deposits and intraneuronal neurofibrillary tangles. These are composed of abnormally phosphorylated and aggregated microtubule-associated tau-protein. An animal model could add great value for understanding AD and would be of considerable importance. Transgenic mouse models expressing human Alzheimer-related proteins (e.g. APP mutations) causing autosomal dominant forms of AD in humans have greatly contributed to an understanding of the neurodegenerative disease. However, because the nonhuman primate model is physiologically much closer to the human, such a model would be particularly valuable in prevention of neurodegenerative diseases.

For cynomolgus monkeys, older than 15 years, amyloid plaques and abnormal tau protein were detected in brain tissue. The degree of plaque burden was variable in these animals. However, amyloid β 1-42 levels in cerebrospinal fluid were elevated in the oldest animals and amyloid plaques showed some molecular similarity to those described in patients. Hyperphosphorylated tau protein was detected in nerve cells and in oligodendrocytes, and Western blot analysis yielded phosphorylation sites comparable to other human tauopathies such as argyrophilic grains disease and Pick's disease. Examples of related amyloid and tau pathology are depicted in Figure 2. These data suggest that aged cynomolgus monkeys may represent useful spontaneous models for age-related neurodegenerative diseases. Whether geriatric cynomolgus monkeys also develop dementia and cognitive dysfunction is currently unclear.

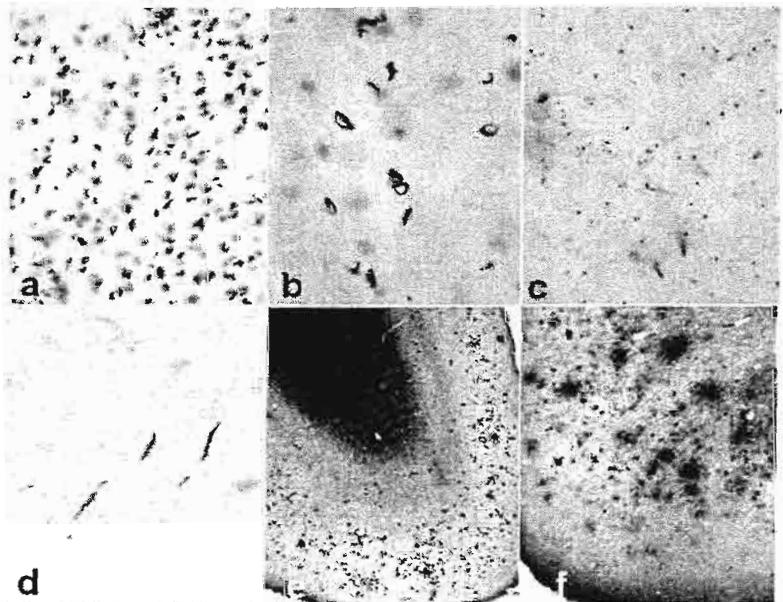
Osteoporosis

Bones are tissues with high metabolic activity undergoing permanent construction and destruction processes. Osteoporosis is a de-

generative disorder of bone in which fractures occur under conditions of normal or mild impact loading. Numerous studies have been performed using adult female monkeys as models for osteoporosis. The natural history of the monkey skeleton has been well documented including the effects of aging on bone mass (slow bone loss in the second decade), reproduction (bone loss due to lactation) and the occurrence of arthritis in both the axial and appendicular skeleton. The physiology of the remodeling systems and its interaction with reproductive physiology have been examined in some detail and show strong similarities to the human skeleton.

Numerous studies have been performed that demonstrate increased bone turnover, osteopenia, and reduced bone strength in ovariectomized monkeys and the model has proven useful for the evaluation of osteoporosis therapies.

Figure 2. Tau protein and β -amyloid peptide pathology in the brain of 20 years old cynomolgus monkeys. a,b) argyrophilic glial fibrillary tangles in the subcortical white matter; c) argyrophilic grains in the dentate gyrus; d) argyrophilic threads in cortical regions; e) vascular amyloidosis and senile plaques in cortex; f) mature and diffuse plaques in cortex.



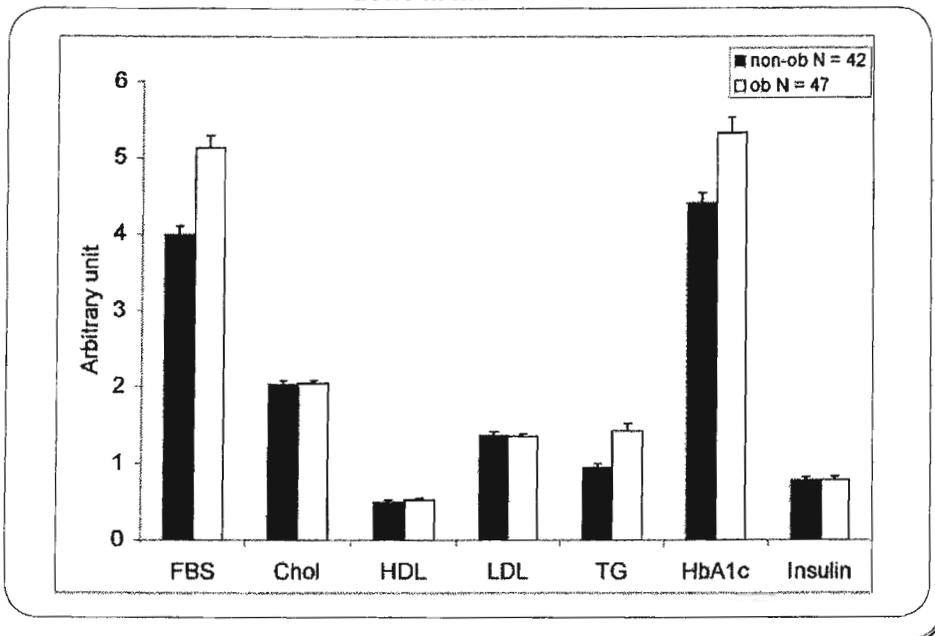
Although much remains to be done and the model has some limitations at the present time it is the best-characterized nonrodent model for the study of osteoporosis. The model may also provide useful for the study of osteoarthritis and other skeletal diseases. Experimentally most studies have involved the use of the ovariectomized adult female monkey for postmenopausal change in bone structure and function. The monkeys experience increased bone turnover, decreased bone mass and a loss of bone strength upon biomechanical testing changes that parallel those seen in postmenopausal women. A variety of potential treatments for osteoporosis have been tested in the model including bisphosphonates, sex steroids, selective estrogen receptor modulators and parathyroid hormone. The cynomolgus monkey is the best-characterized large animal model for osteoporosis research. Spontaneous menopause has been described for the rhesus monkey and recently also for the cynomolgus monkey.

Two forms of degenerative arthritis, calcium pyrophosphate dihydrate crystal deposition disease (CPPD) and osteoarthritis have been demonstrated in the rhesus macaque. Osteoarthritis in the rhesus macaque develops from the age of 5 to 15 years, resulting in structural, chemical, and biochemical changes in joints that closely resemble the features of polyarticular osteoarthritis in humans. This model is particularly suitable for the study of changes in cartilage collagen that may be critically important to the progression of osteoarthritis.

Diabetes

Diabetes mellitus (DM) is a frequent and common endocrine disorder and comprises various forms. Worldwide more than 150 million people suffer from diabetes. In Europe, about 50 million people are assumed to suffer from type-2 DM. Type-1 DM is characterized by the destruction of insulin-secreting pancreatic β cells, leading to an absence of insulin and type-2 is characterized by peripheral defects in the response to insulin. Whereas previously

Figure 3. Serum levels of selected clinical pathology parameters involved in metabolic disorders. Data for insulin are from 42 non-obese (non-ob) animals and 47 obese (ob) animals. Blood was collected from geriatric male and female cynomolgus monkeys at Mauritius and blood analysis was done in Mauritius.

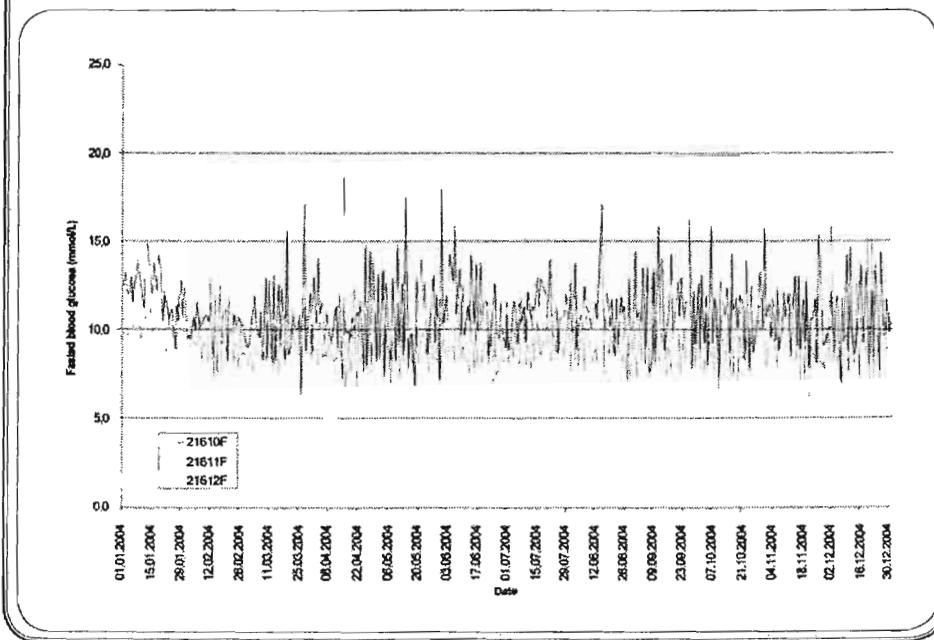


type-2 diabetes was considered typical for aged people, this disease is now also found in middle-aged and young patients. Spontaneous DM has been observed in cynomolgus monkeys exhibiting features reminiscent of human type-2 diabetes.

During an initial screening program in the aged Mauritian cynomolgus monkey colony, 16 out of 222 animals that were screened for fastened blood sugar levels, were in the diabetic range. Recently, comparative analysis of young (below 6 years of age) and geriatric cynomolgus monkeys (older than 20 years) was conducted. Elevated triglyceride and fasted blood sugar levels compared to young animals were evident for the aged animals (Table 2). Comparison of clinical pathology data from obese and non-obese geriatric animals generally older than 13 years is also available (Figure 3). The data show that obese animals have higher fasted blood sugar, acetylated hemoglobin levels and higher triglycerides whereas levels of cholesterol, LDL/HDL and insulin were comparable. Parameters such as fasted blood sugar, acetylated hemoglobin and triglycerides, which differ most in obese and non-obese animals, show the widest range.

The assay methodology for diagnosing metabolic disease and diabetes including glucose tolerance tests is fully established for the cynomolgus monkey model. Figure 4

Figure 4. Daily blood glucose levels in three aged female cynomolgus monkeys. In these animals, feeding was adjusted based upon daily blood glucose levels. At blood sugar levels of 8.4 mmol/L or higher, animals were substituted with 10 U insulin/kg body weight.



provides an example of daily blood glucose monitoring over prolonged periods of time. Daily blood glucose levels are used to control the disease in diabetic monkeys. It is likely that aged cynomolgus monkeys, similar to the already established rhesus monkey paradigm, will provide models for metabolic disease, diabetes and obesity.

Prostate Diseases

The incidence of prostate hyperplasia (i.e., benign prostate hyperplasia BPH) and prostate cancer increase markedly with age and prostate cancer is among the most frequent causes of death in aged men. It is estimated that by 80 years of age, 80% of men will have BPH and 25% will require surgery. As a result of the increasing life span and health status of aging men, prostate cancer and hyperplasia diagnosis and therapy will become increasingly important. Prostate pathology is diagnosed by means of rectal ultrasound of the prostate and by determination of prostate specific antigen from blood which is the best tumor marker for prostate cancer.

The cynomolgus prostate is anatomically very similar to humans and contains a periurethral zone that gives rise to prostate

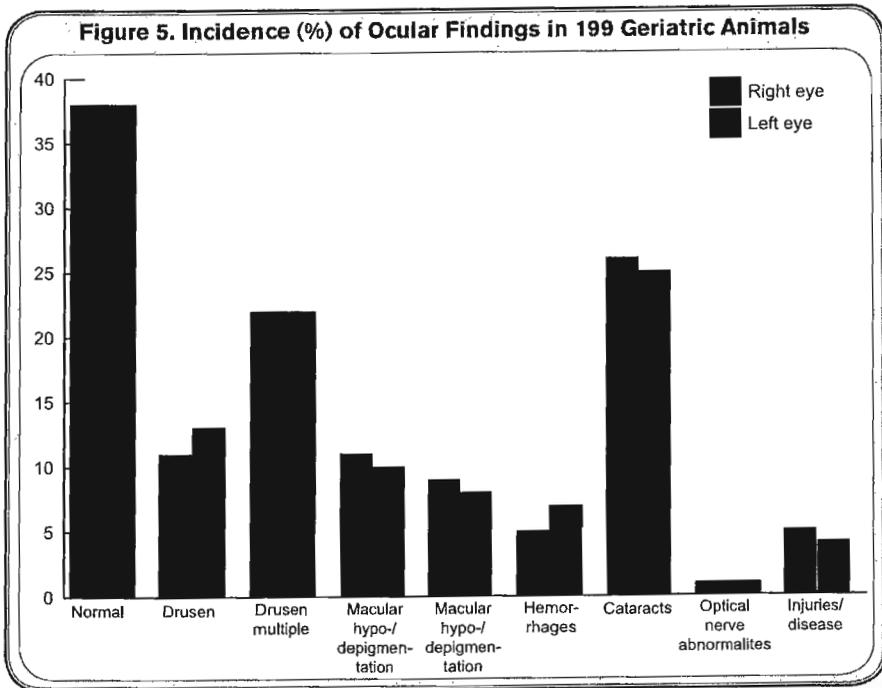
pathology. An ultrasound technique for determination of prostate size and texture in the cynomolgus monkey is available and prostate biopsies can be collected. However, in a recent study of more than 50 aged cynomolgus monkeys no hints for prostate abnormalities could be detected. Screening for prostate-specific antigen did not yield evidence for prostate pathology. It might be that for the spontaneous evolution of prostate disease even older cynomolgus monkeys (20 – 30 yrs) need to be studied.

A Look at Ocular Diseases

Vision capabilities steadily decrease with age and the ocular pathology is related to numerous items including lacerations of glaucoma (e.g., elevation of intraocular pressure), retinopathies, macular degeneration, altered lens function, and ultimately blindness.

In addition, because the eye utilizes many functional principles of the brain and utilizes neurophysiologic and neuropharmacologic mechanisms not present in any other part of the body it is uniquely susceptible to drug side effects. Vision is of paramount importance in primate behavior, and the visual system of the macaque is strikingly similar, both anatomically and functionally, to

Figure 5. Incidence (%) of Ocular Findings in 199 Geriatric Animals



that of humans. Thus, studies of the macaque visual system have generated important new information that is directly relevant to understanding the human visual system and the potential hazardous effects on the system caused by pharmaceutical treatments and compounds.

Primates are excellent models for the study of ocular toxicity covering a wide range of eye parameters such as general morphology, morphology of the cornea, lens, vitreous body, and retina, innervation, blood circulation, iris color, inflammation, electrophysiological properties and age related changes. An array of techniques to qualitatively and quantitatively diagnose and monitor ocular pathology is available for the cynomolgus monkey.

In a recent study, a total of 199 geriatric animals older than 13 years (29 males, 170 females) were screened for ocular pathology by direct and indirect ophthalmoscopy (Figure 5). No ocular findings were detected in 64 animals (32%) while the corresponding incidence in non-aged animals is above 90%. In 123 animals (62%) ocular findings were encountered in both eyes and ocular abnormalities were confined to either the left eye or the right eye in 12 animals (6%). Overall, 68% of geriatric animals presented ocular findings versus less than 10% in non-aged cynomolgus monkeys. The detection of cataract and rubeosis iridis indicate that spontaneous diabetes mellitus in cynomolgus monkey can be associated with ocular lesions that are comparable to human in certain aspects. Disease-associated ocular findings such as cataract, retinopathy and rubeosis iridis are well described for patients with type-2 diabetes mellitus.

Meeting the Challenges of the Gray Revolution

Data from nonhuman primate studies are being gathered from an ever increasing number of diagnostic parameters. The development and adoption of high-tech, noninvasive diagnostic tools (e.g., ultrasound, tomography, magnetic

resonance imaging) is adding to the type of information available from these studies. The amount of knowledge gained through behavioral, physiological, and biomedical studies involving nonhuman primates is immeasurable. Work is always being done to refine the methods and protocols to minimize distress with the ultimate goal of replacing animal use with non-animal techniques wherever possible. However, until advancements in technologies such as genomics are perfected, the responsible conduct of studies utilizing nonhuman primates is critical in the fight for human health. Finally, the demand for efficacious medicines for geriatric diseases will undergo explosive growth during the next 20 years. The same analogy may hold in the development of new therapies for juvenile diseases as well.

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About the author

Gerhard Weinbauer is the director of research services and safety assessment at the Covance facility in Münster, Germany. He holds a PhD in biology and zoology with special expertise on reproductive physiology of primates. Professor Weinbauer also teaches reproductive physiology and medicine at the University of Münster. From 1986 to 2000 he served as head of the Animal Experimentation Department at the Institute of Reproductive Medicine of the University of Münster and managed the institute's primate colony. In 1987 he received the Schoeller-Junkmann Award of the German Endocrine Society, in 1999 the Hamilton-Thorn Prize and in 2001 the European Academy of Andrology Prize. He was board member of the German Endocrine Society and editor of the society's newsletter from 1998 to 2004. Gerhard is the author of more than 200 publications, has edited four books and acts as reviewer for several scientific journals and research supporting institutions.



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European Primate Resources Network (EUPREN)

Initiative of Institutes Performing Research with Non-Human Primates in Europe

This document is based on meetings in Brussels (Okt 1993), Göttingen (Nov 1993), Strasbourg (Dec 1993) and Amsterdam (March 1994) in collaboration with the Directorate General XII of the European Union.

1. Introduction

For the foreseeable future non-human primates will continue to play an important role in biological and biomedical research in Europe. These animal models are essential to study, in vivo, the mechanism of

- Infectious diseases such as AIDS, hepatitis and malaria which are responsible for a high mortality rate in Africa, South America and parts of Asia. With the increased mobility of people, such diseases pose a global threat.
- Autoimmune, immune and neurological diseases such as rheumatoid arthritis, multiple sclerosis and Alzheimer's Disease and Parkinson's Disease. There has been an increase in the number of patients suffering from these diseases, due, in part, to a fast growing group of ageing people in developed countries.
- New therapeutics based on naturally occurring compounds such as lymphokines, new generations of vaccines and gene therapy products, the efficacy of which, for reasons of limited species reactivity can only be investigated in animals that are genetically closely related to humans. In addition, primates are also used as non-rodent species in safety and efficacy studies required by drug regulatory authorities in Europe and elsewhere.

These areas of research as well as fundamental and applied brain, health and fertility research, lead to a total estimated annual requirement of more than 10,000 non-human primates. For ethical and scientific reasons it is generally recognized that wild-caught primates are inappropriate to use and it seems likely that the import of wild-caught animals will be banned by EU in the near future.

To address the requirement to provide non-human primates for research, the primate centres in several European countries have established major facilities for the captive breeding and housing of non-human primates. These facilities are sometimes combined with experimental



17th April 2008.

To whom it may concern.

I am a primatologist at the University of Oxford with extensive experience and a publication record reflecting my research into various aspects of the welfare of primates in experimental and breeding facilities.

I am excited to hear of the possible construction of a longtailed (cynomolgus) macaque breeding facility in Puerto Rico. In particular this facility will be breeding Mauritian origin monkeys that are free of Simian Herpes B Virus and those from BCM are know to be of a high health and psychological status. Among the areas I have researched are the effects of transport on primates. While it is easy to exaggerate the effects of transport it is clear that there are mitigatable effects on the animals, it is easy to see that reducing these effects would be one reason for locating experimental facilities in close proximity to the source of animals others factors include cost reduction, easy transfer of husbandry practices, consistency in animal health care and training, and perhaps most importantly security of supply. It is in this context that many biotechnological and biomedical enterprises are likely to be drawn to locate near breeding facilities that can supply animals that represent good quality scientific models of high health and psychological status. In the context of the proposed facility, Puerto Rico would be well placed to attract such biotechnology and CRO enterprises.

I have visited and assessed primate breeding facilities in, among other places, Mauritius (BCM) and Israel (BFC). I am impressed with the quality of husbandry and welfare of animals at these facilities and have good confidence in the ability of those involved in the proposed facility to transfer those skills and practices to Puerto Rico. Furthermore Puerto Rico has for many years established itself as a centre of excellence for macaque research both in behavioural and physiological disciplines through the work at Sebana Seca and Cayo Santiago. I am sure that the proximity of the Caribbean Primate Research Center and this new facility together with governmental support will only add to the attraction Puerto Rico will hold for the biotechnology and biomedical industries.

A handwritten signature in black ink, appearing to read 'Paul Hones'.

Paul Hones, PhD, BSc.
Primatologist

M.L. & R.E. LAW FIRM

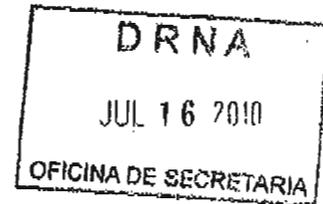
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16 de julio de 2010

Hon. Daniel J. Galán Kercadó
Secretario
Departamento de Recursos Naturales y Ambientales
P. O. Box 366147
San Juan, Puerto Rico 00936



Re: Enmienda al Permiso de Importación de Animales Núm. 2010-IC-O-VS-PVS15-SJ-00416-0502-2010.

Estimado Secretario Galán:

El pasado 25 de mayo, el Departamento de Recursos Naturales y Ambientales (en adelante "DRNA") expidió el permiso de referencia. Luego de revisar el mismo proponemos las siguientes enmiendas al permiso para su consideración. Las enmiendas aquí presentadas tienen el propósito de aclarar algunas condiciones y dar cumplimiento a las mismas balanceando la viabilidad de las operaciones de la empresa con el deber del DRNA de velar por el cumplimiento de la política pública del Estado en materia de conservación de vida silvestre.

Enmiendas al Permiso Número 2010-IC-O-VS-PVS15-SJ-00416-0502-2010'

Previo a entrar en las condiciones del permiso señalamos que en el epígrafe del Permiso aparece el término "Expira" pero consigna la fecha de emisión. Suponemos que es un error clerical y que debe ser la fecha de emisión. Por lo tanto, se debe cambiar para que lea "Fecha de expedición: 26 de mayo de 2010". De otra parte, para que no haya dudas ni controversias sobre el tipo de permiso, entendiéndose que es un permiso de importación y exportación para fines científicos, en el epígrafe en la parte de "Tipo de Permiso" debe decir: "Importación y Exportación para Fines Científicos".

Ahora bien, atendiendo específicamente las condiciones del permiso, solicitamos las siguientes enmiendas:

Condición 1.5. - La redacción se presta a confusión ya que el límite de 4,500 individuos durante la vigencia del permiso es aplicable a la importación, no a la exportación. Para que esté claro, se propone la siguiente enmienda, que no altera el propósito de la condición:

¹ Se utilizara el mismo sistema de enumeración del permiso actual para facilitar la identificación de las condiciones a enmendarse.

Se autoriza a Bioculture PR, Inc. a importar y exportar para fines científicos, primates de la especie *Macaca fascicularis*. Entendiéndose que el número de animales a importar no excederá la capacidad autorizada por el Departamento de Agricultura de los Estados Unidos (USDA) de jaulas para mantener los animales; y la importación de animales para procrear no será más de cuatro mil quinientos (4,500) individuos durante el término de vigencia de este Permiso, según se desglosa a continuación:...

Condición 1.7.1. - Recomendamos la siguiente redacción de este inciso para propósitos de claridad sin que se afecte el resultado porque para todos los efectos, los autorizados por ley se refiere a entidades académicas de educación universitaria dedicadas a la investigación científica en Puerto Rico:

Será ilegal vender, ceder o transferir los animales en cuestión a otra persona o entidad, dentro del territorio de Puerto Rico, excepto a las entidades autorizadas por ley dedicadas a investigación científica en Puerto Rico. De Bioculture PR, Inc. realizar cualquier venta, cesión o transferencia a cualquiera de éstas, deberá notificarlo al DRNA dentro de un término de treinta (30) días, a partir de la fecha en que se realice la transacción. La notificación deberá incluir el número de primates que hayan sido vendidos, cedidos o transferidos.

Condición 1.7.4. - De la misma no surge en que momento se debe entregar el certificado veterinario. Sugerimos que el certificado médico deberá conservarse en las facilidades de Bioculture y estar disponibles para producirse en casos de inspecciones o por solicitud oportuna del DRNA.

Condición 1.7.8 - La descripción de las medidas de seguridad se presta para confusión ya que el sistema de verjas, según el plano aprobado por ARPe incluye una verja electrificada con detectores de movimiento. Se debe mencionar también el sistema de triple seguro en las jaulas. Sugerimos el siguiente lenguaje:

La instalación contará con máxima seguridad. Lo anterior incluye, pero no se limita, a lo siguiente: verja alrededor del perímetro de las jaulas que deberá estar electrificada, jaulas con triple seguro para acceder a los animales, guardias de seguridad las veinticuatro (24) horas, alarmas contra escape y detectores de movimiento en la verja del perímetro conectado al sistema de alarmas.

Condiciones 1.7.10 y 1.7.11 - Bioculture está en disposición de adquirir el seguro de responsabilidad pública con el límite impuesto de un millón de dólares y los respectivos endosos sin la imposición de una fianza. Es evidente que la responsabilidad de los daños que causen los animales está cubierta por el seguro de responsabilidad pública,

por ello, no es necesaria la fianza en este renglón. El interés público no se vería afectado por este cambio pues habrá una aseguradora respondiendo, además de Bioculture. En resumidas cuentas, lo que se propone es que los límites escalonados descritos en el art. 1.7.12 sea el de la póliza de seguros, ya que ésta cubriría la responsabilidad pública. De lo contrario, sabemos que tal fianza no solo resultaría redundante, sino que, al no existir compañías dispuestas a vender tal producto, dicho requisito impediría que Bioculture pueda operar en Puerto Rico. está disponible y no haría viable la operación.

Condición 1.7.12. Modificar este artículo para atemperarlo a los cambios antes descritos sobre el seguro de responsabilidad pública y al establecimiento de un Fondo Especial según se sugiere a continuación:

Bioculture está dispuesto a establecer un Fondo Especial de ciento setenta mil dólares (\$170,000)² para asegurar aquellos gastos relacionados a la captura de animales en casos de fuga y manejo de los animales en casos de abandono o quiebra de Bioculture. Dicha suma de dinero se consignará en una cuenta PLICA y los fondos podrán ser retirados por el Gobierno de Puerto Rico, el DRNA o el Departamento de Agricultura según se describe a continuación:

1.7.12.1 - En aquellos casos relacionados a la fuga de animales (incluyendo casos de desastres naturales, causa mayor o negligencia atribuible a Bioculture), Bioculture deberá llevar a cabo gestiones para la captura inmediata del animal. Si en veinticuatro (24) horas no logra su captura, deberá notificarlo al DRNA. Aquellos gastos que incurra el DRNA para la captura de los animales deberá ser reembolsado a través de la compañía aseguradora y/o Bioculture. En caso de que la compañía aseguradora o Bioculture no cumplan con el reembolso de los gastos, el DRNA podrá desembolsar la cantidad correspondiente del Fondo Especial. Bioculture proveerá personal experto en captura de primates para asistir al DRNA.

1.7.12.2 - En caso de un abandono o quiebra de Bioculture donde no se dispusieran de los animales, se podrá utilizar el dinero en el Fondo Especial para que el DRNA lleve a cabo las gestiones necesarias para disponer de los mismos.

Condición 1.7.14 - Esta condición plantea un serio problema por implicar una renuncia a derechos constitucionales. Además, debe haber una razonabilidad en la intervención

² Esta cantidad esta basada en los más de 20 años de experiencia de Bioculture en el manejo y captura de animales. A pesar de que durante la existencia de la compañía no ha ocurrido fuga de animal alguno, Bioculture ha llevado operaciones de captura de los *Macaca Fascicularis* y conoce el trabajo que conlleva la captura de los mismos así como el equipo necesario para ejecutar dicha operación. [Ver Anejo 1] También se conocen los costos de disponer de los animales por razones de eutanasia. [Ver Anejo 2].

de forma que se eviten abusos o arbitrariedades. A tales efectos, el artículo debe leer:

El DRNA por conducto de la Unidad de Vida Silvestre del Cuerpo de Vigilantes, podrá en cualquier momento y sin previo aviso, entrar a las instalaciones de Bioculture PR, Inc., para inspeccionar que las condiciones de este Permiso se estén cumpliendo. Entendiéndose que dichas visitas para inspección no serán arbitrarias y caprichosas ni con frecuencia irrazonable. El empleado que realice la inspección, si desea acceder las áreas de los animales, deberá cumplir con las medidas de cuidado y seguridad requeridas por Bioculture y el Departamento de Agricultura de los Estados Unidos (USDA). Mediante la firma de este documento Bioculture PR, Inc. renuncia expresa pero limitadamente a cualquier reclamo de derecho contra registros irrazonables en cuanto a las inspecciones que puedan llevarse a cabo por el DRNA para verificar el cumplimiento con las condiciones de este Permiso pero con ningún otro registro.

Condición 1.7.16. - Los cambios que se sugieren es que se amplíe el tiempo que tiene Bioculture para capturar el animal evadido. Aunque con el diseño de los edificios de la operación y las medidas de seguridad que se habrán de tomar, no se prevé que hayan fugas, lo cierto es que cuatro (4) horas para capturar el animal constituye un término arbitrario e irrazonablemente corto. Además, no es necesario reclamar directamente al seguro si Bioculture tiene un plazo razonable para reembolsar los gastos. Por ello, recomendamos modificarlo para que lea:

En caso de que se escape cualquier primate por la razón que fuere, Bioculture PR, Inc. será responsable del rastreo y captura del mismo. Si dentro de veinticuatro (24) horas del escape, Bioculture PR, Inc. no ha podido atrapar el primate, tendrá la obligación de notificar este hecho al DRNA, quién se encargará de la captura del mismo y recobrará los costos de tal operación de conformidad con lo establecido en el art. 1.7.12, ante. De Bioculture PR, Inc. incumplir con la notificación, el Permiso podrá ser revocado.

Condición 1.7.18 - En este caso lo correcto sería el someter copia certificada de la póliza de seguro de responsabilidad pública. Debe, por tanto, leer:

Bioculture PR, Inc. será responsable de someter copia certificada de las pólizas de seguro de responsabilidad pública, por las cantidades establecidas anteriormente, con treinta días de anticipación a la fecha en que entre en vigor el segundo, tercero, cuarto y quinto año de vigencia del Permiso. El incumplimiento con esta disposición podrá dar lugar a la revocación de este Permiso.

Condición 1.7.20. - Se repite y/o contradice lo provisto en los artículos 1.7.1.7 y 1.7.13.
Para mayor claridad debe leer:

Este Permiso tendrá vigencia y operará en pleno vigor en la fecha de su expedición, condicionado a que en los próximos treinta (30) días de haber sido expedido, Bioculture PR, Inc. haga entrega al DRNA de copia certificada de las pólizas de seguro de responsabilidad pública y de la fianza de cumplimiento requerida para cada año de vigencia de este Permiso.

Con las enmiendas aquí contenidas Bioculture podrá llevar a cabo su operación satisfactoriamente garantizando el interés público de maximizar la seguridad del ambiente y los ciudadanos de Puerto Rico.

Estamos disponibles para discutir las enmiendas solicitadas a su conveniencia o aclarar cualquier duda sobre las mismas.

Sin otro particular quedo de usted.

Respetuosamente,


Emil Rodríguez Escudero

ANEJO 1

Bioculture (Puerto Rico) Inc.
 Pueblita Carmen, Box HCO2 - 4350
 Guayama
 Puerto Rico 00784

Document BCM.PR.02.07

Animal Trapping Team

Bioculture has over 25 years experience in the trapping of wild (feral) monkeys on the island of Mauritius.

Trapping of monkeys requires a great knowledge of animal biology and behaviour.

The best trapping team should be comprised of an animal care taker who is very familiar with animal behaviour and an assistant to help place and maintain traps.

Equipment needed:

- Binoculars
- 4 individual traps that are baited with food and are triggered by the animal itself. These are placed near animal with its favorite food inside.
- Nets for animal trapping.
- Tranquilizing darts which can be fired or blown at the animal.
- Radio communication
- 4x4 vehicles.

A daily operation cost for a professional trapping team is about 200\$

A monthly operation cost of a professional trapping team is about 5000\$

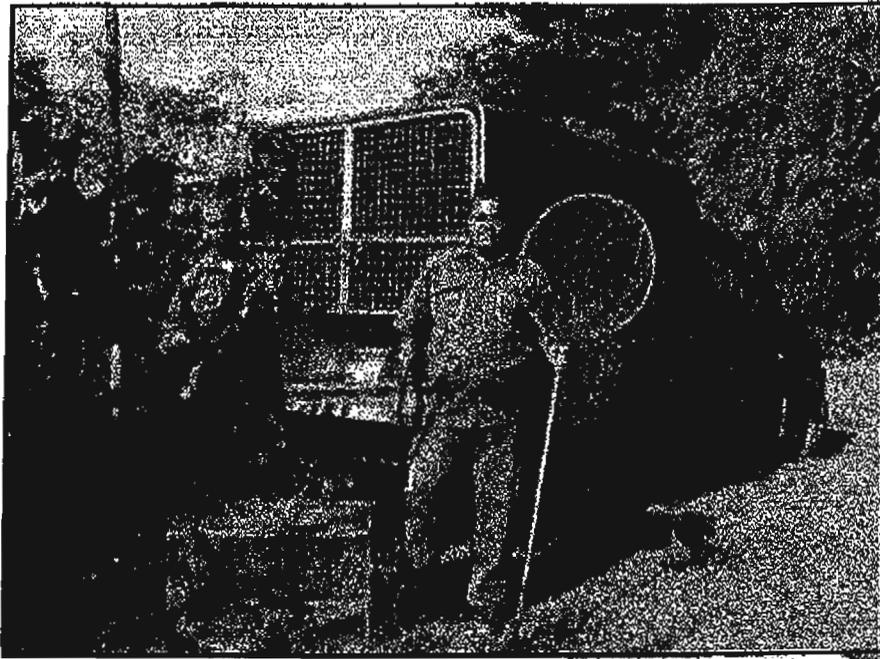
A trapping team will cover a very large area per day searching for animals.

It should however be remembered that:

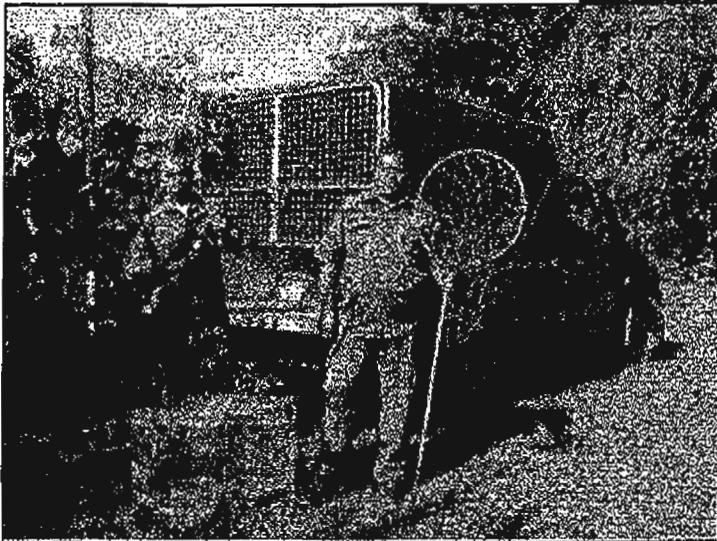
1. Animals as a general rule will not venture far from the farm site in the event of an escape, and are thus easily recaptured.
2. Bioculture has invested very significantly in cage design and infrastructure and site fencing to prevent escapes.

Owen Griffiths
 Managing Director.





Trappers, Net, dart gun and transport cage





BIOCULTURE
ORGANIZACION SIN FINES DE GANANCIA

Bioculture (Puerto Rico) Inc.
 Pueblita Carmen, Box HCO2 - 4250
 Guayama
 Puerto Rico 00784

Document BCM.PR.01.07

Euthanasia of animals

- ⇒ Animals are euthanized by an injection of concentrated anesthetic.
- ⇒ The Material is given based on the body weight and the quantity for adult animal is 4-5 kg of body weight (like a cat).
- ⇒ The price of a dose to anesthetic to euthanize one animal is between \$1 and \$2.
- ⇒ Procedure to be carried out by veterinarian with animal technician.
- ⇒ A veterinarian can euthanize a very large number of animals per day if needed.
- ⇒ In animal shelters veterinarians can euthanize hundreds of animals per day.
- ⇒ We estimate the cost for one animal with its disposal to be not more then \$50 and the price will drop sharply if the quantity is much bigger.

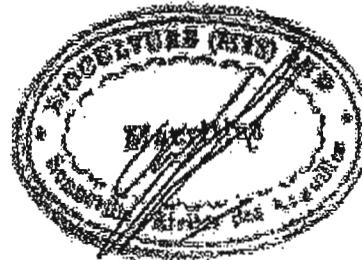
Cost table for an euthanasia procedure.

Time/manpower for procedure: 1 animal technician and 1 veterinarian for 2 mins (time taken for procedure)

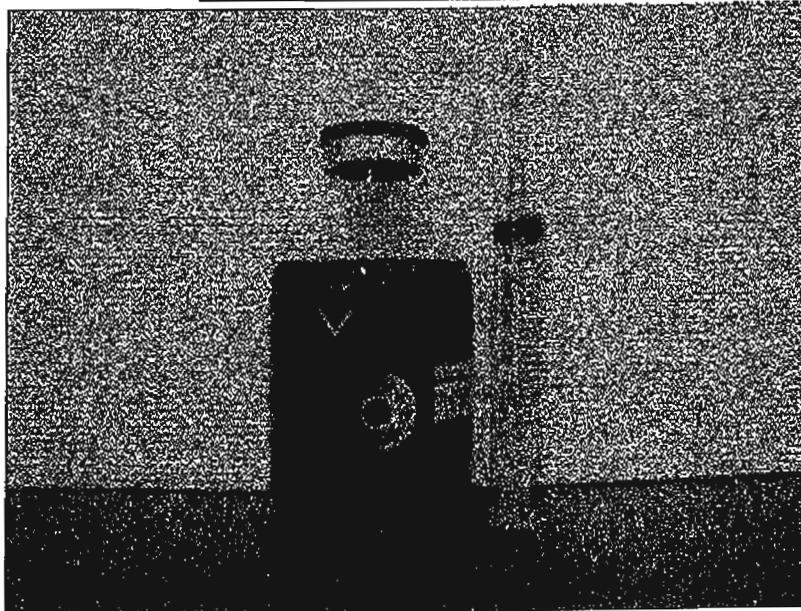
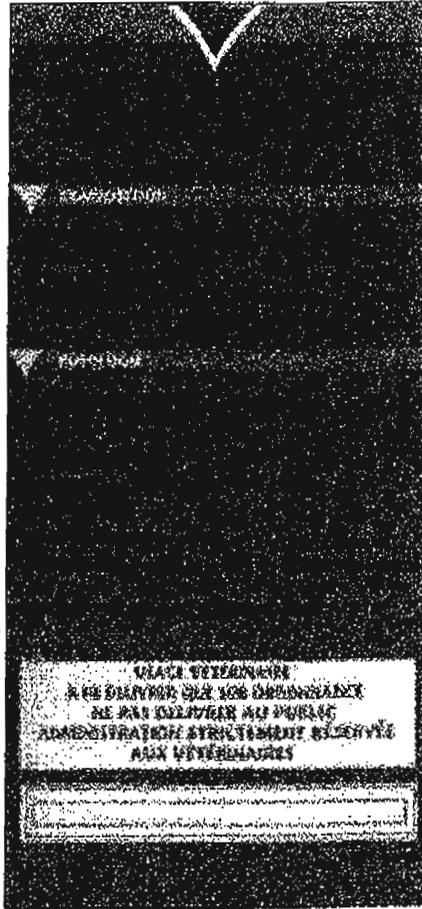
PROCEDURE: EUTHANASIA

	AMOUNT	COST (\$)	
TIME	2 mins		
MANPOWER			
Handler/Technician	1		
Veterinarian	1		
ITEMS			
Syringe+ Needle	2	0.50	
Latex Gloves	8	0.30	
Ketamine	0.4 ml	0.30	
Dolethal	4 ml	1.00	
Disposal Bag	1	0.50	
TOTAL		\$2.60	

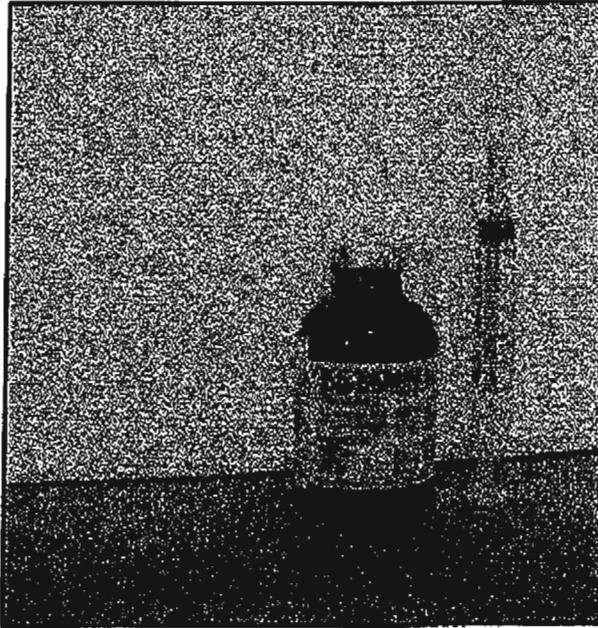
All calculations based on a 4 kg animal
 Disposable items plus medicine costs based on \$US costs in Mauritius.



Euthanasia drugs



Tranquilizers



RETARNO 1000 mg

Indicaciones:
Tranquilizante y sedante. Se utiliza para el tratamiento de la ansiedad.

Modo de usar:
Se debe tomar con agua, preferentemente en el momento de acostarse, una vez al día. El uso prolongado puede generar dependencia y síndrome de abstinencia. Evitar el alcohol y otros medicamentos que actúen sobre el sistema nervioso central.

Contraindicaciones:
Embarazo, lactancia, depresión, alcoholismo, insuficiencia renal o hepática, miastenia gravis.

Efectos secundarios:
Somnolencia, mareos, debilidad, pérdida de peso, disminución de la actividad sexual, dependencia y síndrome de abstinencia.

Precauciones:
Evitar el alcohol y otros medicamentos que actúen sobre el sistema nervioso central. Evitar la conducción de vehículos y el uso de maquinaria pesada.

Interacciones:
Puede potenciar los efectos de otros medicamentos que actúen sobre el sistema nervioso central.

Forma farmacéutica:
Comprimidos.

Presentación:
Caja con 100 comprimidos.

Indicaciones:
Tranquilizante y sedante. Se utiliza para el tratamiento de la ansiedad.

Modo de usar:
Se debe tomar con agua, preferentemente en el momento de acostarse, una vez al día.

Contraindicaciones:
Embarazo, lactancia, depresión, alcoholismo, insuficiencia renal o hepática, miastenia gravis.