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ヌマカ属（アシマダラヌマカ）の幼虫および蛹の呼吸器の走査電子顕微鏡による形態観察

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昭和63年8月18日受付／昭和63年12月26日受理

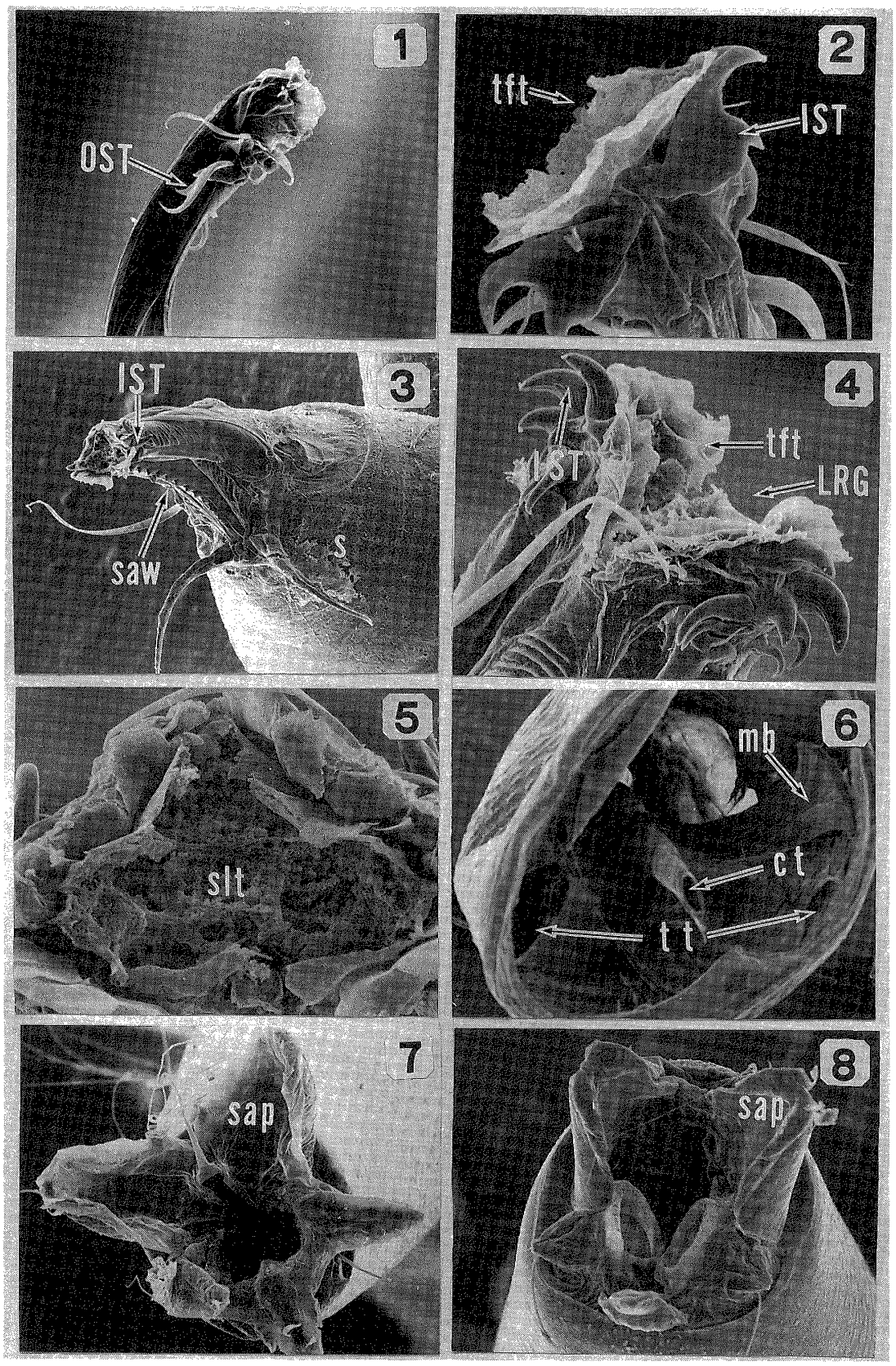
緒　言

Mansonia 属の蚊は，マレー糸状虫 *Burugia malayi* の媒介蚊として知られ，疫学的に非常に重要な蚊である（Samarawickrema, 1968; Laurence, 1960）。この蚊の幼虫および蛹の水中における特殊な呼吸法に関しては，他の *Culex* 属や *Aedes* 属などとは異なり，幼虫や蛹の生息する水域に自生する水生植物の根や茎に呼吸管，または呼吸角を挿入して直接植物組織内に含まれる酸素を呼吸するものと考えられていた（Laurence, 1960）。しかし，著者は，以前 *Mansonia* 属の幼虫および蛹を生体のままホールスライドグラス内に遊泳させ，光学顕微鏡によって観察した結果を報告した（Iwaki,1984）。即ち，水中に静止している幼虫の呼吸管先端を静かに刺激すると，呼吸管先端内部から千鳥型の爪と非常に薄い膜状組織が突出すること，また，この組織が水中の溶存酸素を呼吸する呼吸鰓（larval respiratory gill＝LRG）であることを示した。さらに，蛹の呼吸角先端部については，水生植物の根や茎に呼吸角先端を挿し込み体を固定するために鋭く尖った角状を呈していること，また，植物組織から酸素を吸収するための穴は開口していないばかりでなく，呼吸角先端部を包む網目状組織の呼吸角鰓（pupal respiratory gill＝PRG）を有し，これによって呼吸すると考えられることを報告した（Iwaki, 1984）。今回は，走査電子顕微鏡を用いて，幼虫の呼吸管および蛹の呼吸角にみられる微細構造について観察した。1令幼虫の呼吸管先端部の両側にカギ状の爪と，先端内部から一対の千鳥型の爪および薄い花冠状の膜組織が突出していること，そして2令から4令幼虫の呼吸管先端部にはさらに多くの爪と花冠状の膜組織とそれに包まれて特殊なスポンジ状の構造をもった組織がみられた。一方，蛹の呼吸角の最先端は鋭く尖った角状を呈し，先端部側面に毛細血管状の管によって作られたレース状組織がみられ，いずれも，水中の溶存酸素を呼吸するための呼吸鰓であることが認められた。また，呼吸管および呼吸角内部の微細構造についても観察した結果から，幼虫および蛹の体内の血液循環に関する経路についての知見を得たので考察を加えて報告する。

材料および方法

観察に供した *Mansonia*（*Mansonioides*）*uniformis*（Theobald）アシマダラヌマカの幼虫，および蛹は，タイ国・マヒドール大学熱帯医学部医用昆虫学教室において飼育した個体（Supat *et al.*, 1982）であり，比較のために用いた *Culex tritaeniorhynchus summorosus*（Dyar）コガタアカイエカおよび *Aedes japonicus*（Theobald）ヤマトヤブカの幼虫および蛹は，京都市内で採集し飼育して得た個体である。試料は，あらかじめ70%アルコール液に保存し，標本作成時に80%から100%アルコールに置換した後，自然乾燥させた（Matsuo *et al.*, 1972）。この試料をミニポール濾紙上に少量の接着剤を付けて固着させ，更にこれをアルミニウム試料台に両面テープによって

京都女子大学家政学部生物学教室


1
OST
2
tft
IST
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IST
saw
s
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IST
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LRG
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slt
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mb
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sap
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sap

固着させた。

この試料を日立E101 10N蒸着用スパッターにより金蒸着し，日立S-530 走査電子顕微鏡を用い，加速電圧10～15KVのもとに観察した。なお，幼虫および蛹の呼吸器の各部名称は，Harbach and Knight (1980) およびIwaki (1984) を適用した。

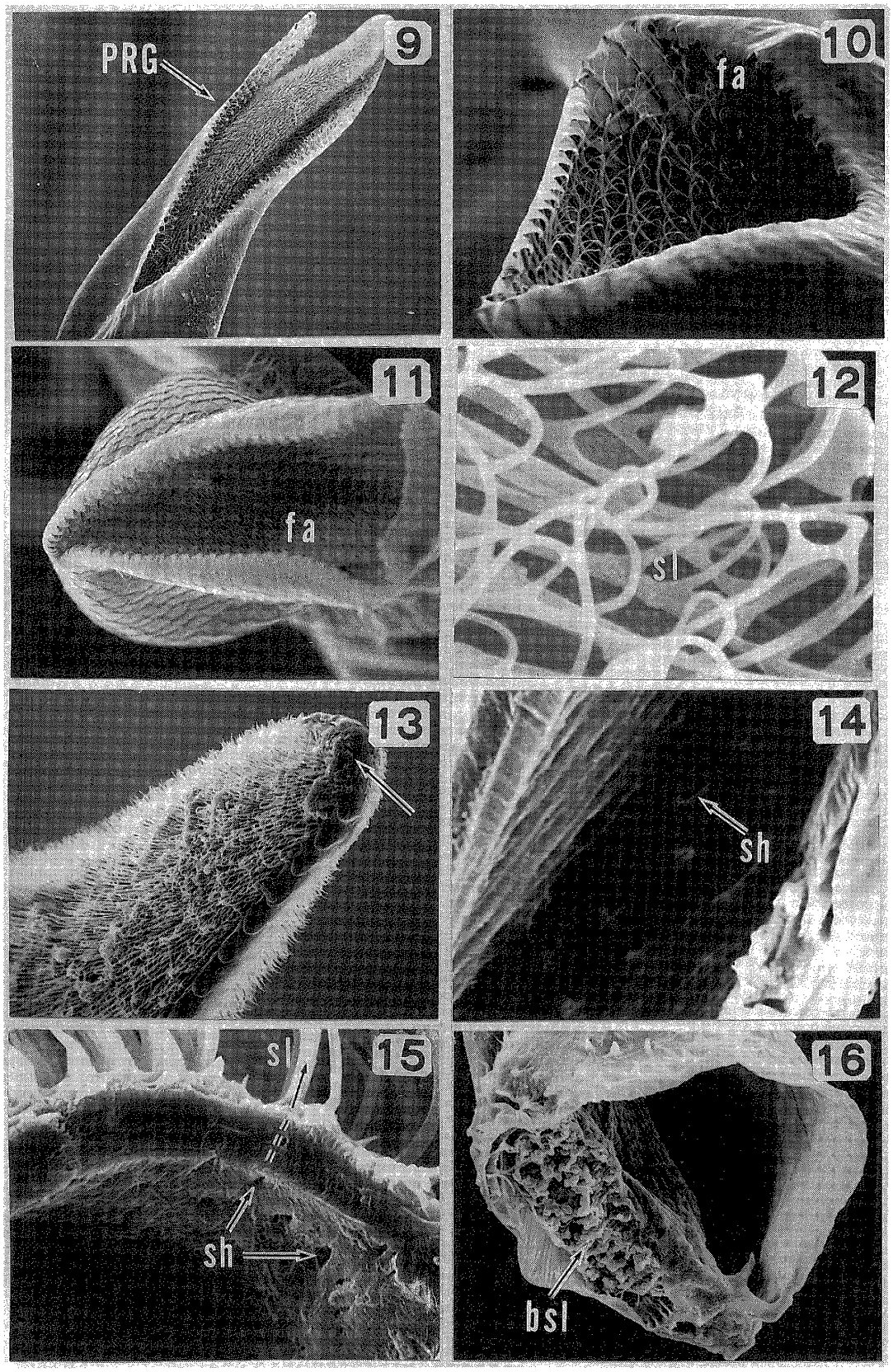
結果および考察

1．幼虫の呼吸管の観察結果

Mansonia 属のアシマダラヌマカ1令幼虫の呼吸管先端は，図1のごとく，*Culex* 属のコガタアカイエカおよび *Aedes* 属のヤマトヤブカの呼吸管と類似し，円筒型を呈して細長いが，呼吸管先端部の両側にカギ状の爪（outer spiracular teeth＝OST)が一対みられる。そして，この1令幼虫が水生植物の根や茎に固着した場合，図2に示されるごとく，呼吸管先端内から一対の千鳥型の爪（inner spiracular teeth＝IST）と薄い花冠状の膜組織（thin filmy tissue＝tft）が突出する。アシマダラヌマカの幼虫の呼吸管は，2令から4令へと令の進行にしたがって円錐型に変化するが，呼吸管先端部には顕著な変化がみられないので，本報告では，2令・3令幼虫については省き，4令幼虫について記述する。図3は，4令幼虫の呼吸管先端を上側面より観たところであるが，ノコギリ状歯（saw）とISTがみられ，図4に示すごとく水生植物の根や茎に体を固着した場合に呼吸管先端内から突出する多数のISTとtftが認められた。さらに，先端中心部には，図5に示すごとくtftに包まれたスポンジ状組織(sponge-like tissue＝slt)がみられる。また，円錐型の呼吸管の中央部を横に切断した内部には，図6の矢印に示すごとく，中心部に1本の太い管（cylindrical tube＝ct）と両側面に2本の筋束（muscular band＝mb)，そして呼吸管内壁に試料を作成した際に少し扁平に変形しているが，気管状の管（thick trachea＝tt）が左右一対あることが，それぞれ観察された。

2．幼虫の呼吸管に関する考察

Culex 属や *Aedes* 属の幼虫の呼吸管は細長い管状を呈し，その先端は図7および図8におのおの示されるごとく，水面に浮上して呼吸管先端を水表面に突出したときはsapを開き空気中の酸素を直接呼吸管内にとり込み，また，水中を遊泳する場合はsapを閉じ気管内に水が侵入することがない仕組みになっている（Gillett, 1971)。しかし，*Mansonia* 属の幼虫の水中における呼吸法は，Bates (1949)によれば，幼虫は水生植物の根や茎の組織を呼吸管側面に付属するsawによってくだき，その中に呼吸管先端を挿し込み植物組織内に含有する酸素を呼吸するので，幼虫は水面にはほとんど現われることはないと述べている。またGillett (1971) は，幼虫の行動について，普通の蚊と同様に後方向に水中を遊泳し，強く水生植物の根や茎に呼吸管を挿し込む。このとき体をリズミックに動かしsawを使って植物組織に穴を開け細い呼吸管先端を挿し込み，同時に6対の爪をもって体を固着させると述べている。さらにKettle (1984) は，呼吸管は扁平で短かい円錐型を呈し，堅い頑丈な弁をもち，水生植物の空気を含有している組織にまで呼吸管を挿し込み呼吸することが可能な形をもっていると述べている。しかしながら，今回走査電子顕微鏡（SEM）を用いてアシマダラヌマカの幼虫の呼吸管各部の微細構造について観察した結果，1令幼虫の呼吸管には水生植物の根や茎に穴をあけるためのノコギリ状歯は全くみられず，また呼吸管先端が開口した場合には内部からISTおよびtftが突出することが観察された。このことから，1令幼虫は呼吸管先端部のOSTによって水生植物の根などに懸垂して体を固着させ，同時に呼吸管先端内部より突出するLRGによって水中の溶存酸素を呼吸すると考えられる。さらに高令幼虫の呼吸法については，水生植物の根や茎に呼吸管先端を強く触れISTおよびOSTを用いて体を固着させ，呼吸管先端内部より突出したtftに包まれたLRGによって呼吸することが示唆された。このようにしてLRGから水中の溶存酸素がとり込まれた血液の循環については，すでにIwaki (1984) が光学顕微鏡による生体観察の結果について述べているごとく，左右2本に分岐したttを通り幼虫体内に


9
PRG
10
fa
11
fa
12
sl
13
14
sh
15
sl
sh
16
bsl

運ばれ，体の各部に酸素を供給したあとは体内の中心部にあるポンプ状の管内に集められ，それにつながるct内を通り再び呼吸管先端のLRGにもどる循環経路をもっている。なお図6の側面にみられるmbは，ctを前後に移動させctの先端にあるLRGを呼吸管先端内から出し入れさせるためにあり，また，同時にISTを強く振動させて水生植物の根や茎に押しあてISTを植物組織に懸けるために使われる筋肉である。

3．蛹の呼吸角の観察結果

アシマダラヌマカの呼吸角は，バナナ状で図9に示すごとく呼吸角最先端は鋭く尖った角状で，図10のコガタアカイエカや図11のヤマトヤブカの蛹にみられるようなトランペット型とは全く異なった形状を呈している。そして，先端部は図12のごとく毛細血管状の管（slender loop＝sl）がレース状に組み合された鰓状組織PRGによって被われている。また，呼吸角の最先端は水生植物の根や茎に直接挿入し体を固定するための形を呈し，図13に示されるごとく植物組織から酸素を吸収するような穴は全く開口していない。さらに，呼吸管先端部を縦に切断した内部には，図14のごとく多数の小穴が内壁に点在していることが観察され，同部分の横断面には図15の矢印に示すごとく，内壁の小穴から外壁のPRGに連絡していることが明らかとなった。また，呼吸角基部の横断面には，図16のごとく，内壁に細い管の管束(bundle of slender loop＝bsl）の部分と空洞の2つの部分に分かれていることが認められた。

4．蛹の呼吸角に関する考察

*Culex*属や*Aedes*属などの蛹の呼吸角はトランペット型を呈し，*Culex*属では水中に潜る場合，図10のごとく呼吸角先端内に細毛があり，その部分に気泡を保ち呼吸管内に水が侵入しないように仕組まれている。そして，再び水表面に浮上し呼吸する際には，先端部を水表面につき出し水の表面張力がはたらき管内には水が流入しないようになっている。一方*Aedes*属では，呼吸角先端部に図11に示されるごとくバルブを有し，水中に潜る場合はバルブを閉じ，水表面に浮上した場合には開口することを，著者は蚊の飼育中に観察し，またGillett (1971) もそのことを述べている。しかし，*Mansonia*属の蛹の特殊な呼吸法については，Bates (1949)によれば蛹の呼吸角先端を水生植物の根や茎に強く挿入することによって体を植物体に固定すると同時に，植物組織に含まれる酸素を呼吸角先端から吸収することによって生活するため，羽化するまで静止状態を続け水面には浮上しないと述べている。また，Gillett (1971) は，蛹の呼吸角先端はドリル状を呈し水生植物の根や茎に穴を開け，その部分に呼吸角先端を挿し入れ呼吸するとともに体を固着させると述べている。しかし，今回の著者による走査電子顕微鏡での観察によれば，蛹の呼吸角先端には細かい毛があり，鋭く尖っていてドリル状の構造はみられないばかりでなく，最先端には空気を吸収するための穴も全く開口していない。即ち，呼吸角先端は，単に水生植物の根や茎に体を固着させる為に尖っていて，実際に呼吸に関与する部分は毛細管状のslからなるレース状組織であると考えられる。このことから，体内から呼吸角に送り出された血液は，呼吸角の中心部を通り呼吸角先端内部に達し，多数開口しているshからslのレース状のPRGに送られ，そこで水中の溶存酸素を血液内にとり込み，再び呼吸角内のbslを通り体内にもどる循環経路をもつことが示唆された。

結　論

*Mansonia*属の蚊の幼虫および蛹は，*Culex*属や*Aedes*属とは異なり，特殊な呼吸法を営むことが知られている。Bates (1949)，Gillett (1971)，そしてKettle (1984) らのそれぞれの著書には，*Mansonia*属の幼虫および蛹は，水生植物の根や茎に呼吸器の先端を挿し込むことによって植物組織中に含まれる酸素を呼吸すると述べられている。しかしながら，これらの記述は，いずれも肉眼的または光学顕微鏡的観察による知見にすぎない。今回，著者は走査電子顕微鏡を用いて幼虫の呼吸管および蛹の呼吸角の微細構造をくわしく観察した結果，次のような結論を得るに至った。

1．幼虫の呼吸法については，1令幼虫の場合，

呼吸管外壁に付属する気管外爪（OST）によって水生植物の根や茎に懸垂して体を固着し，2令から4令幼虫の場合は，呼吸管先端を水生植物に触れさせ気管内爪(IST)を用いて植物体に付着して体を固定させる。そして，実際の呼吸は，すべての令を通して，呼吸管先端内から突出する呼吸管鰓（LRG）によって水中の溶存酸素を取り込むことによって行う。

2．蛹の呼吸角全体はバナナ状を呈し，キチン化して鋭く尖った最先端を水生植物の組織に強く挿し込み，体を固着させる。そして，呼吸角先端部に附属する毛細血管状の管がレース状になった呼吸角鰓（PRG）によって，水中の溶存酸素を呼吸すると考えられることが構造上示唆された。

稿を終るに臨み，本研究の資料の提供に御協力下された，タイ国・マヒドール大学熱帯医学部医用昆虫学教室の Dr. Supat Sucharit 教授並びに教室員各位に深謝する。

尚，本稿の内容は，第36回日本衛生動物学会大会（1983年10月）において発表した。

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LARVAE

Photo 1-6 *Mansonia uniformis*:

Phote 1 Side view of the closed tip of siphon in lst instar larva, ×1,000
2 Dorsal view of the opened tip of siphon in lst instar larva, ×2,000
3 Anteralside view of the opened tip of siphon in 4th instar larva, ×300
4 Dorsal view of the opened tip of siphon in 4th instar larva, ×1,000
5 Ventral view of the opened tip of siphon in 4th instar larva, ×3,000
6 Inner part of the siphon of 4th instar larva, ×1,000
7 Anterior view of the siphon of 4th instar larva (*Culex tritaeniorhynchus summorosus*), ×400
8 Anterior view of the siphon of 4th instar larva (*Aedes japonicus*), ×400

PUPAE

Photo 9 and 12-16 *Mansonia uniformis*:

Photo 9 Side view of the tip of trumpet, ×300
10 Anterior view of the trumpet of pupa (*Culex tritaeniorhynchus summorosus*), ×400
11 Anterior view of the trumpet of pupa (*Aedes japonicus*), ×400
12 Respiratory "gill" of the structure of a slender loop, ×6,000
13 Ventral view of the tip of trumpet, ×500
14 Cross section of the tip part of trumpet, ×3,000
15 Vertical section of the tip part of trumpet, ×3,000
16 Cross section of central part of trumpet, ×500

ABBREVIATIONS

OST: outer spiracular teeth
LRG: larval respiratory gill
s: siphon
saw: saw
IST: inner spiracular teeth
tft: thin filmy tissue
slt: sponge-like tissue
sap: spiracular apparatus
ct: cylindrical tube
tt: thick trachea
mb: muscular band
PRG: pupal respiratory gill
sl: slender loop
sh: small hole
bsl: bundle of slender loop
fa: filter apparatus

MORPHOLOGICAL OBSERVATION OF LARVAL AND PUPAL RESPIRATORY APPARATUS OF *MANSONIA* (*MANSONIOIDES*) *UNIFORMIS* (THEOBALD) (DIPTERA, CULICIDAE) BY SCANNING ELECTRON MICROSCOPE

MISAO IWAKI

Received August 18 1988/Accepted December 26 1988

The respiratory organs of *Mansonia uniformis* larvae and pupae were minutely observed by scanning electron microscope (SEM). Crown-shaped, gill-like, thin filmy and sponge-like tissues were observed at the tip of the siphon of the lst and 4th instar larva. Similarly, characteristic structure consisting of fine, slender loop (like capillary blood vessels) was observed at the tip of the pupal trumpet. The auther suggested that immatures of *Mansonia* take in water soluble oxygen with the respiratory "gills" which exist at the tip of the siphon of the larva and at the tip of the trumpet of the pupa.

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Trypanosoma brucei gambiense 感染マウス血清中のIL-2産生抑制物質

陶　　道，日笠　　穣，宮本彦四郎
山田　和彦，小松　俊憲，新家　荘平

昭和63年9月5日受付/平成元年1月24日受理

はじめに

トリパノソーマ原虫は，アフリカ睡眠病を引き起こす病原体である。この原虫が感染すると，原虫の表面抗原に対する特異抗体が産生されるが，トリパノソーマは抗原変異により，この宿主の防御反応より逃れることが知られている。また，トリパノソーマ感染時には，非特異的免疫抑制状態になることが報告されている。この抑制の成因について，免疫細胞の機能低下，抑制細胞の存在，或はインターロイキン-2（IL-2）活性の低下等が考えられている。最近になって，トリパノソーマと同様に免疫抑制を起こすマラリア原虫に感染すると，マウスの血清中にIL-2活性を抑制する，いわゆるIL-2インヒビターが上昇することが報告された（Lelchuk *et al.*, 1985)。このIL-2インヒビターの上昇は，マラリア感染時のIL-2産生能の低下の原因の1つと考えられ，したがってマラリア感染時の免疫抑制に関与している可能性が示唆されている。

今回の実験において著者らは，トリパノソーマ感染による免疫抑制には，IL-2インヒビターが関与しているかどうかを明らかにするため，*Trypanosoma brucei gambiense* の感染時における血清のIL-2インヒビター活性，正常脾細胞のIL-2産生に対する影響ならびに脾細胞のIL-2産生能を調べた。

材料と方法

1）実験動物

BALB/cマウスとC57BL/6マウスは日本チャールス・リバー株式会社より購入した。感染実験には，6週齢の雌性マウスを用いた。

2）原虫および感染方法

Trypanosoma brucei gambiense（以下，Tbgと略する）はマウスで長期継代されたWellcome株を用いた。Tbg原虫の分離は，Lanhamの方法に準じた（Lanham *et al.*, 1970)。すなわち，感染マウスより心採血し，DEAEセルロースカラムを通過させて血液成分より分離した後，リン酸緩衝生理食塩水（pH 7.0）で3,000 rpm，10分間の遠沈により3回洗浄したものを実験に用いた。マウスへの感染は，Tbg原虫2×10^4個を腹腔内に投与することで行った。

3）脾細胞浮遊液の作成

Tbg感染後1日，2日，3日目のマウス，または正常マウスより，脾細胞を無菌的に摘出し，単細胞浮遊液を作成した。細胞浮遊液は，トリパンブルーにて生細胞数を数えて，5×10^6個/mlに調整した。培養液は5×10^{-5}Mの2-メルカプトエタノール，10%牛胎児血清(FCS)を含むRPMI-1640を用いた。

4）IL-2産生能の測定

感染マウス脾細胞によるIL-2産生能の測定は，Lelchukらの方法に準じて，脾細胞浮遊液の5×10^6/mlにConcanavalin A（Con A, Miles社）を最終濃度2 μg/mlになるように加え，37℃，

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5％炭酸ガス下に24時間培養した後，その培養上清を回収し，IL-2活性測定用のサンプルとした（Lelchuk *et al.*, 1984）。

IL-2の活性は，Gillisらの方法に準じてIL-2依存性のCTLL-2細胞の増殖反応を測定することで求めた（Gillis *et al.*, 1978）。即ち，段階希釈したサンプル100 μl を96穴マルチプレートに入れ，5×10^4/ml のCTLL-2細胞を100 μl 加え，37℃, 20時間培養後，^{3}H-thymidine（アマシャム・ジャパン株式会社）0.5 μCi/well を加え，さらに4時間培養した後，セルハーベスタ（Semi-automatic multiple cell harvester, Labo Science Co., Ltd.）でグラスファイバーフィルター（ラボマッシュ用フィルター）上に細胞をハーベストした後，^{3}H-thymidineの取り込みを液体シンチレーションカウンター（Packard 460 CD, USA）で測定した。

5）IL-2インヒビターの測定

1．血清内に含まれるIL-2インヒビター活性の測定は，Lelchukらの方法に従った（Lelchuk *et al.*, 1985）。即ちTbg感染1，2，3日目のマウスの血清を培養液で段階希釈し，この希釈した血清50 μl を1×10^4個のCTLL-2細胞浮遊液100 μl および100 unit/ml のrecombinant IL-2（rIL-2，シオノギ製薬）50 μl を含む96穴のマイクロプレート中で20時間の培養後，^{3}H-thymidineを0.5 μCi/well 加え，さらに4時間培養した後，^{3}H-thymidineの取り込みの減少によりIL-2インヒビター活性を測定した。

2．IL-2産生に対する抑制は，Tbg感染3日目のマウスの血清が正常脾細胞のIL-2産生を抑制するか否かで判定した。すなわち正常脾細胞（5×10^6/ml）を最終濃度2 μg/ml のCon Aで刺激し，同時にその培養系に上記の血清を総容量の1％，2％，4％および8％の濃度で加え，24時間後の培養上清中のIL-2活性を，CTLL-2細胞の増殖度により測定することで判定した。

結　果

1）Tbg感染脾細胞におけるIL-2産生能

Tbg感染マウス脾細胞のCon A刺激に対するIL-2産生能を調べた。その結果，図1で示すようにTbg感染3日目のBALB/cおよびC57 BL/6マウス脾細胞のIL-2産生能は正常マウスに比べて低下していることが明らかとなった。しかしながら，感染1日目，2日目では，IL-2産生能はコントロールと較べて，明らかな低下を示さなかった。

2）Tbg感染マウス血清中のIL-2インヒビター活性

Tbg感染3日目の血清中のIL-2インヒビター活性は，BALB/cおよびC57 BL/6マウスの何れの系統においても正常血清とほぼ同様であり，感染によるIL-2インヒビター活性の増強は，認められなかった（図2）。感染1日，2日目のIL-2インヒビター活性も3日目と同様に正常血清と比較して上昇していなかった。

3）正常脾細胞のIL-2産生能に対する影響

感染血清の正常脾細胞のIL-2産生への影響をみた。図3に示すように，感染3日目の血清は，8％の濃度で正常血清より明らかな抑制作用を示した。

考　察

トリパノソーマ感染によって起こる免疫抑制には，様々な機序が存在することが多くの研究者によって報告されてきた（Ackerman *et al.*, 1976; Person *et al.*, 1978; Romos *et al.*, 1979; Cunningham *et al.*, 1980; Maleckar *et al.*, 1983）。特にT細胞の機能に関しては，細胞内寄生原虫である *Trypanosoma cruzi* あるいは細胞外寄生原虫の感染において，マウスの脾細胞のCon Aに対する増殖反応の低下や，IL-2産生能の低下が報告されている（Bellan *et al.*, 1983; Alcina *et al.*, 1985）。またIL-2を *T. cruzi* 感染マウスに投与することによって，原虫に対する特異的な免疫反応の増強，虫血症の減少，マウスの延命効果が報告されている（Choromanski *et al.*, 1987）。これらの事実は，T細胞の機能低下が，*T. cruzi* または *T. brucei* 感染時の免疫抑制現象の重要な要因になっていることを示唆している。しかし，*T. b. gambiense* 感染については，いまだ報告されていない。

近年，Lelchukらはトリパノソーマと同様に免

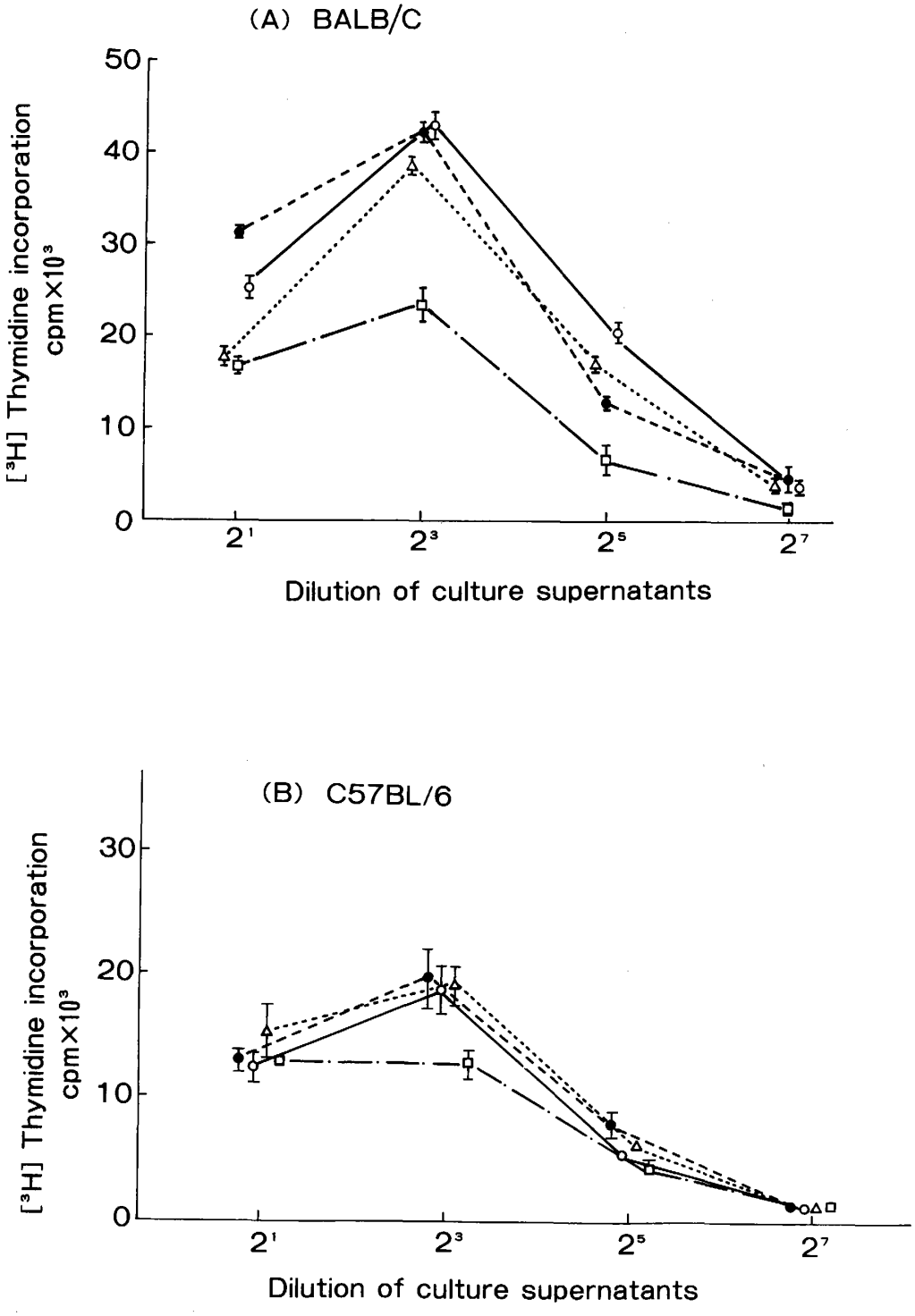

Figure 1 Titration curve of IL-2 production. Spleen cells collected from normal (●) and Tbg-infected BALB/c (A) and C57B/6 (B) mice 1(○), 2(△) or 3(□) days after infection were cultured with Con A for 24 hr and IL-2 activity of the culture supernatants were assayed using IL-2 dependent CTLL-2 cells. Results indicate mean±S.D. of three experiments.

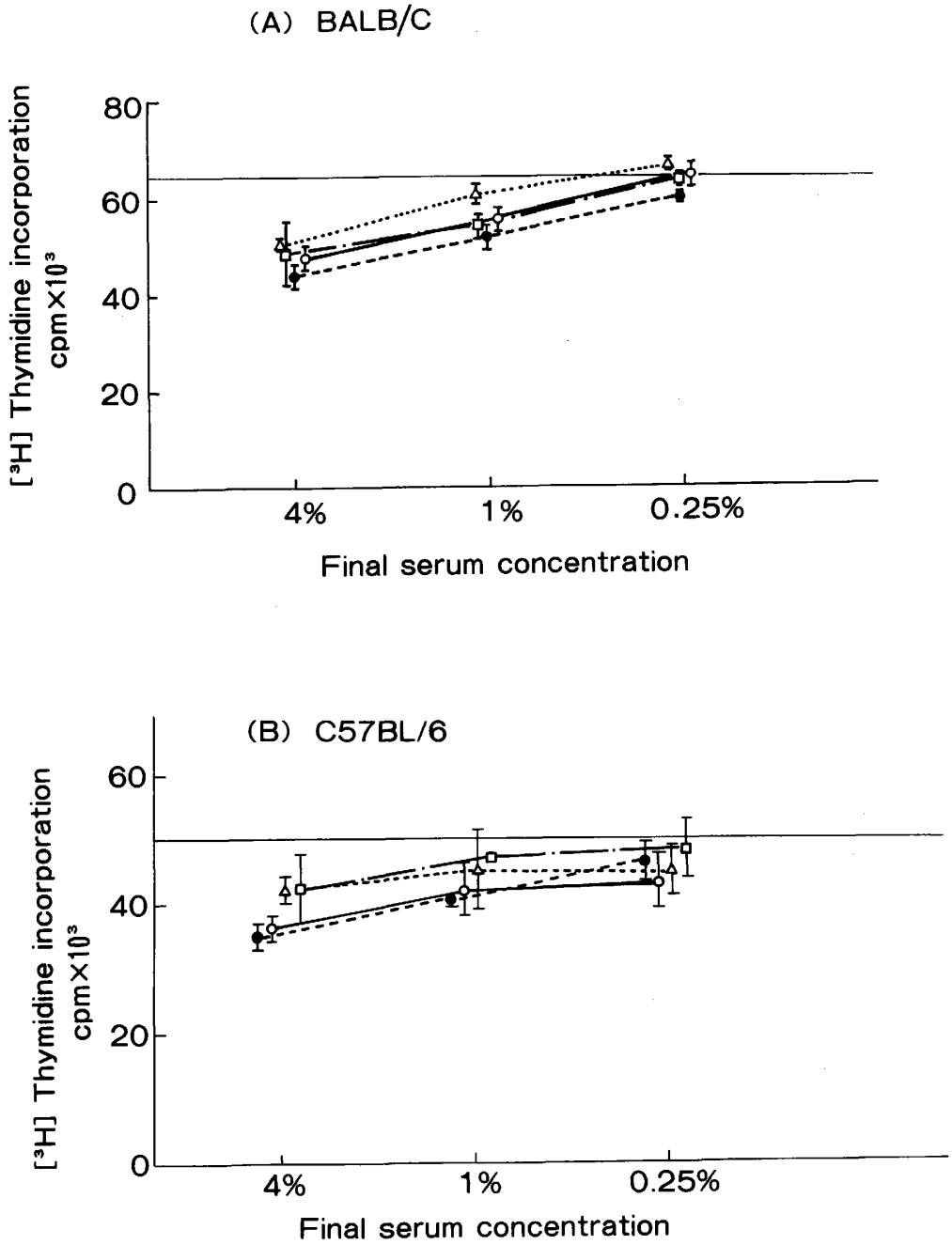

Figure 2 Inhibition of the activity of recombinant IL-2.
Fifty μl of serially diluted normal mouse serum (●) or sera sampled from mice 1(○), 2(△) or 3(□) days after infection were added, together with 50 μl of 100 unit/ml rIL-2 to 1×10^4 CTLL-2 cells in 100 μl of culture medium. After 24-hr culture, proliferation of CTLL-2 cells were measured by ^{3}H-TdR incorporation. In the control, culture medium was added instead of serum and c.p.m. level were 64,600±6,578(A) or 49,758±4,127(B). Results indicate mean±S.D. of three experiments.

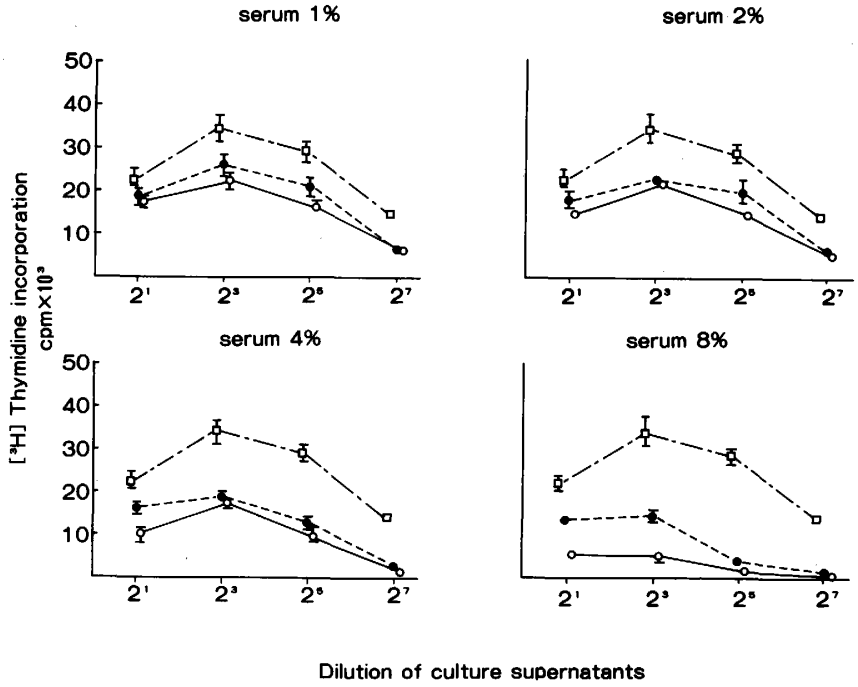

Figure 3 Inhibition of IL-2 production. Con A activated normal spleen cells (5×10^6/m*l*) were incubated with serially diluted serum from either BALB/c normal mice (●) or mice infected for 3 days with Tbg (○). After 24-hr-culture, the remaining activity of IL-2 in the cell-free supernatants were assayed using CTLL-2 cells. Results indicate mean±S.D. of three experiments. Maximum activity of IL-2 released in the absence of additional serum (□) is shown as a control of each condition.

疫抑制を起こすマラリア感染について，マウス血清中のIL-2インヒビターの変動を報告した（Lelchuk *et al.*, 1985)。彼らによると，IL-2インヒビターは無胸腺マウスや無菌条件下で飼育されたマウス血清中には存在しないが，正常マウス血清中に存在し，マラリア（*Plasmodium yoelii*）感染時に上昇するという。

今回の実験では，*T. b. gambiense* 感染におけるT細胞の機能を検索するためにマウス脾細胞のIL-2産生能を調べたところ，感染3日目に著しく低下していた。そこで，IL-2産生の低下にいかなる抑制機構が存在しているかを明らかにするために感染3日目のマウス血清中のIL-2インヒビターを測定した。その結果感染3日目のマウス血清は，8％の濃度で正常脾細胞のIL-2産生を明らかに抑制した。しかしながらCTLL-2細胞のIL-2依存性増殖に対しては，抑制反応を示さなかった。このことから *T. b. gambiense* 感染におけるIL-2抑制因子は，IL-2と競合的に作用するものではなく，IL-2産生を抑制するものと考えられた。

IL-2インヒビターはHardtら（Hardt *et al.*, 1981)の報告に，Lyt-2，3⁺のsuppressor T細胞より分泌されてT細胞の機能調節の一環を担っているとされ，マウス血清中では，分子量5万のタンパク質とも言われているが，その詳細はいまだ不明である。ヒトでは，血清中にprostaglandinの様なIL-2活性を抑制する物質も含まれており（Rappaport *et al.*, 1982)，かならずしもIL-2インヒビターといわれる単一の物質が存在するかについては異論もある（Lelchuk *et al.*, 1987)。さらにこれら，いわゆるIL-2インヒビター活性は寄生虫の種や感染時期，寄生部位によって様々な活性の変化をきたす可能性も考えられる。しか

しながら，いずれにせよ8％の感染マウス血清がIL-2産生を抑制したことから *in vivo* では，この感染マウス血清が，かなりの免疫抑制作用に関与している可能性が示唆された。

トリパノソーマ感染による免疫抑制の機序には単なるIL-2活性の変化以外に，抗原提示細胞の機能低下やsuppressor macrophageの出現も報告されている（Bagasra *et al.*, 1981; Corsini *et al.*, 1977; Eardley *et al.*, 1977)。こういった免疫反応の低下と同時に，interferonやNK活性が上昇しているという報告もある（Hatcher *et al.*, 1980)。トリパノソーマ感染による免疫反応の変化は，免疫抑制といった単純な一方方向の免疫現象ではなく，様々な免疫現象が複雑にからみあったものであり，これらの液性因子や免疫調節細胞の総合的解明が，トリパノソーマ感染防御への手がかりの1つになると考えられる。

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IL-2 PRODUCTION INHIBITORY ACTIVITY OF THE SERA IN *TRYPANOSOMA BRUCEI GAMBIENSE* INFECTED MICE

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African trypanosome infections result in severe immunosuppression. Previous studies have shown that this suppression is due, at least in part, to suppressor cells (T cells and macrophages) or impairment of IL-2 production.

In the present study, the effects of *Trypanosoma brucei gambiense* infection on serum levels of IL-2 inhibitor and Concanavalin A (Con A) induced IL-2 production of spleen cells were investigated. The results showed the ability of infected spleen cells to produce IL-2 was markedly reduced and IL-2 production of normal spleen cells was inhibited by the infected sera. But the IL-2 dependent CTLL-2 cell proliferation was not inhibited by those sera. These results suggest that depletion of IL-2 production during *T. b. gambiense* infection may be due to the increase of an inhibitor of IL-2 production.

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ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) IN THE DETECTION OF HEPATITIS-B SURFACE ANTIGEN IN BLOOD DRIED ON FILTER PAPER

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Abstract: In an attempt to find a convenient method for the seroepidemiological study of HBsAg in developing countries, ELISA using blood samples dried on filter paper was evaluated. The results of this method were in complete agreement with both those obtained on original sera by RIA and with those obtained on sera by ELISA. We did not experience any false positive or false negative results. In this study the sample dried on filter paper was eluted by 1 m*l* of PBS (pH 7.2). Dilution rates were calculated by the calibration line of each serum. Dilution rates were between 0.041 and 0.082. The coefficient of variation of absorbance value of 10 samples of each case was between 5.0% and 14.8%, which indicated an acceptable intratest reproducibility of this method. In addition, storage at 20°C, 20°C with desiccants and 3°C for up to 21 days did not alter the sensitivity and specificity of this method. This method will be useful for the seroepidemiological study of HBV in developing countries.

INTRODUCTION

While conducting a seroepidemiological study in developing countries, researchers often encounter the following difficulties:

1. Collecting blood samples
   It is often difficult to take venous blood from newborns, children, anemic patients, pregnant women and other adults who are not accustomed to such a procedure
2. Storage and transportation of blood samples
3. Economical problems
4. Technical problems

The planning method may not be available for local conditions. These difficulties are magnified when the population being studied is spread over a large area or living far away from research centers.

To overcome these difficulties, blood samples dried on filter paper have been used to detect markers of various infectious diseases like Onchocerciasis (Korenaga *et al.*, 1983; Tada *et al.*, 1985), Japanese encephalitis (Yamamoto *et al.*, 1985) and Rubella (Sander *et al.*, 1985). As far as HBsAg is concerned, the filter paper method has been applied to the detection using radioimmunoassay (RIA) and reversed passive hemagglutination (RPHA) (Farzadegan *et al.*, 1978; Villa *et al.*, 1980; Kitazumi *et al.*, 1981; Zhuang *et al.*, 1982), and

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some seroepidemiological studies have been carried out by this method (Prince *et al.*, 1981; Werner *et al.*, 1985).

Enzyme-linked immunosorbent assay (ELISA) which was invented by Engvall and Perlmann (1971) has been used to detect various pathogenic agents because of its high sensitivity, specificity and rapidity. Ukkonen *et al.*, (1977) demonstrated the usefullness of ELISA to detect HBsAg. Compared to RPHA, ELISA is a more sensitive method. Also, ELISA does not have any regulations which RIA have about radioactive agents. In this study we intended to detect HBsAg in blood dried on filter paper using ELISA.

Materials and Methods

1. Collection of samples

Ten HBsAg positive cases were selected from out-patients of the 1st Department of Internal Medicine, University of Occupational and Environmental Health (UOEH). Ten HBsAg negative cases were selected from student volunteers of UOEH. All cases were retested by a commercial RIA kit (Ausria, Abbott Laboratories) on HBsAg. Twenty blood samples dried on filter paper were collected from each HBsAg positive case and ten samples from HBsAg negative cases.

2. Filter paper

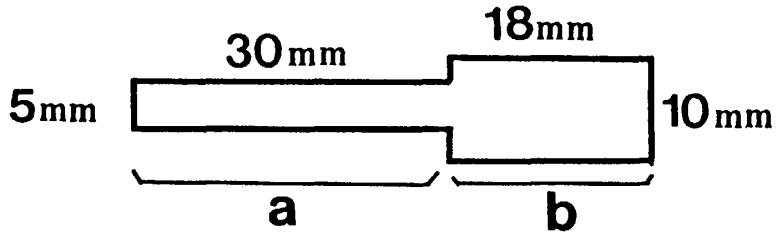
In this study we used a commercial blood sampling paper (Type I, Toyo Roshi, Ltd., Japan). This paper, which was developed by Nobuto (1970), is composed of two areas, an absorbing area and a diffusion area (Fig. 1). The absorbing area is allowed to absorb as much blood as possible. An excess of blood is allowed to be diffused into the diffusion area. In this way the amount of blood contained in the absorbing area is considered to be almost constant (0.1 m*l* which is equal to 0.04 m*l* of serum). Whole blood was collected by this type of paper and air-dried for 2 days at room temperature with the absorbing area up, avoiding direct sunlight. The blood samples dried on filter paper were stored in small polyethylene envelopes under 3 different conditions, 20°C, 20°C with desiccants and 3°C to be tested after 7, 14 and 21 days from collecting.

3. Elution

The absorbing area was cut into 4 portions and placed in a test tube. Then 1 m*l* of PBS (pH 7.2) was added and the test tube was incubated at 37°C for 3 hr with vibration for elution.

4. ELISA procedure

In this study we used a commercial kit, IMMUNIS HBsAg EIA (Sanko Junyaku Co., Ltd.). The procedure was as follows: 1) 0.1 m*l* of eluate was added to each well. 2) The



[a] blood absorbing area
[b] blood diffusion area

Figure 1 Blood sampling paper.

plate was incubated for 2 hr at 37°C. 3) After the reaction, the eluate in well was aspirated. 4) The wells were washed 5 times with wash fluid supplied by the manufacturer. 5) 0.1 m*l* of horse radish peroxidase conjugated antibody (mouse anti-HBs*) was added to each well. 6) The plate was incubated for 2 hr at 37°C. 7) The wells were emptied by aspiration. 8) The wells were washed 5 times by wash fluid. 9) 0.1 m*l* of freshly prepared substrate solution (o -phenyl-diamine, OPD) was added to each well. 10) The plate was incubated for 30 min at room temperature in the dark. 11) The enzymatic reaction was stopped by adding 0.05 m*l* of 4 N H_2SO_4. 12) The plate was read by an autoreader (EAR340, Australia S.L.T. Co., Ltd.). In addition to the above tests the original sera were tested by ELISA to compare them with the results obtained from blood dried on filter papers. The results were interpreted according to the manufacturer's recommendation, that is, positive ELISA values were $>2.5\times$ (mean ELISA values of negative control sera).

RESULTS

1. Comparison of results obtained by RIA and our method

Table 1 shows the comparison of results obtained by RIA and this method. The results are in complete agreement with those obtained on original sera by RIA, indicating acceptable sensitivity and specificity of this method.

2. Comparison of results obtaind by ELISA in serum and in whole blood dried on filter paper

Table 2 shows the comparison of results obtained by ELISA in serum and in whole blood dried on filter paper. The results were in complete agreement.

3. Dilution rate

Theoretically speaking, the dilution rate is 0.04 when the sample dried on the absorbing area of the filter paper was eluted by 1 m*l* of PBS (pH 7.2). In this study we made calibration lines of the original sera to calculate the dilution rate from the absorbance value of each sample (Fig. 2). The results are shown in Table 3. The dilution rates were between 0.041 and 0.082.

4. Reproducibility

The intratest reproducibility of this method was assessed by 10 blood samples dried on filter paper 10 times for each case within the same test. The results of 6 cases are shown in Table 4. Four cases were excluded from discussion because their absorbance values overflowed. The coefficients of variation (C.V.) were between 5.0% and 14.8%. According

Table 1 Comparison of results for detection of HBsAg obtained by RIA for sera and those obtained by ELISA for blood dried on filter paper

		Sera tested by RIA	
		Positive	Negative
Sera dried on the filter paper tested by ELISA	Positive*	200	0
	Negative	0	100

* Case in which ELISA value was $>2.5\times$ (Mean ELISA value of negative controls)

Table 2 Comparison of results obtained by ELISA for detection of HBsAg in serum and blood dried on filter paper

		Sera tested by ELISA	
		Positive	Negative
Sera dried on the filter paper tested by ELISA	Positive*	10	0
	Negative	0	10

* Case in which ELISA value was>2.5×(Mean ELISA value of negative controls)

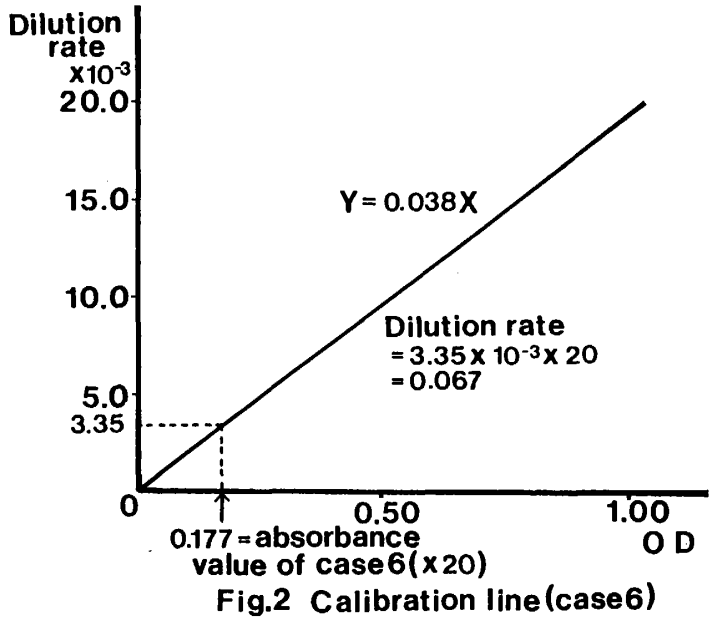


Fig.2 Calibration line(case6)

Table 3 Dilution rate calculated from calibration line

Case No.	1	2	3	4	5
Dilution rate	0.069	0.082	0.041	0.044	0.063
Case No.	6	7	8	9	10
Dilution rate	0.067	0.069	0.047	0.044	0.055

Mean: 0.058 SD: 0.014

to the explanatory note of IMMUNIS HBsAg EIA, the C.V. of the original ELISA procedure are 7.7% and 9.6%. The results indicated the acceptable intratest reproducibility of this method.

5. Influence of storage

Samples dried on filter paper were tested after 7, 14 and 21 days from the first test. The

Table 4 Intratest reproducibility

Case No.	3	5	6	7	9	10
Sample 1	1.814	0.287	1.327	0.386	1.775	2.316
2	1.652	0.293	1.360	0.343	1.782	2.446
3	1.686	0.264	1.327	0.535	1.902	2.608
4	1.514	0.297	1.302	0.539	1.794	2.844
5	1.386	0.283	1.562	0.398	1.924	2.555
6	1.469	0.294	1.355	0.401	1.969	2.256
7	1.700	0.253	1.732	0.432	1.790	2.561
8	1.808	0.278	1.739	0.416	1.728	2.853
9	1.407	0.294	1.427	0.414	1.640	2.327
10	1.586	0.285	1.314	0.393	1.534	2.294
Mean	1.602	0.283	1.445	0.426	1.784	2.506
SD	0.155	0.014	0.171	0.063	0.131	0.218
C.V.(%)	9.68	4.95	11.83	14.79	7.34	8.70

results are shown in Table 5. None of the HBsAg positive samples became negative or vice versa. Also, there were no remarkable differences in the three storage conditions. These results might indicate that HBsAg is stable at least for 2 weeks in a dried condition.

Discussion

It seems to be apparent from our results that HBsAg is stable in a dried condition and ELISA using the filter paper method is reliable and reproducible. As for sensitivity and specificity, we have not experienced any false negative or false positive results. These results were in concord with other reports about the filter paper method (Farzadegan *et al.*, 1978; Villa *et al.*, 1980; Kitazumi *et al.*, 1981; Zhuang *et al.*, 1982). Farzadegan *et al.* (1978) described the following remarks regarding the merits of this simple method:

1. Blood samples may be obtained from newborn infants, children, anemic patients, pregnant women and other adults from whom it is difficult to obtain venous blood.
2. Samples are dry, light, easy to post, and cannot be broken or spilt.
3. Samples are easy to store; they take up little space, and remain stable for a long time.
4. The greater ease and economy of this procedure will be useful in large scale population screening projects.

Now we are planning to detect other HBV markers by ELISA using blood samples dried on filter paper in order to apply this method to the hepatitis B surveillance program in developing countries. It is well-known that hepatitis B infection is very common in developing countries, especially Subsaharan Africa, Southeast Asia, China, and the Western Pacific islands. According to WHO Technical Report (1983), in low endemic areas, such as North America and Western Europe, prevalence of HBsAg is 0.2-0.5%; in intermediate endemic areas such as Eastern Europe and Southwest Asia, the prevalence rate is 2-7%; in high endemic areas such as those written above, the prevalence varies from 6-15% and a high prevalence of chronic liver disease, including hepatocellular carcinoma was also shown. It has been proven that the HBV carrier state is strongly connected with chronic liver disease.

Table 5 Change of absorbance values by storage under 3 different conditions

		HBsAg Positive				HBsAg Negative			
		Days							
Sample		0	7	14	21	0	7	14	21
Sample	1	over	over	over	over	0.039	0.024	0.026	0.027
			over	over	over		0.022	0.026	0.031
			over	over	over		0.025	0.022	0.030
Sample	2	over	over	over	over	0.027	0.030	0.030	0.028
			over	over	over		0.029	0.029	0.030
			over	over	over		0.028	0.025	0.025
Sample	3	1.602	1.636	1.715	1.756	0.046	0.044	0.041	0.036
			2.214	1.875	1.881		0.039	0.041	0.042
			1.813	1.524	1.526		0.039	0.031	0.035
Sample	4	over	over	over	over	0.026	0.025	0.028	0.022
			over	over	over		0.022	0.020	0.019
			over	over	over		0.022	0.025	0.025
Sample	5	0.283	—	—	—	0.046	0.044	0.041	0.041
			—	—	—		0.044	0.042	0.043
			—	—	—		0.040	0.041	0.039
Sample	6	1.445	1.709	1.317	—	0.029	0.029	0.030	0.030
			1.555	1.494	—		0.030	0.028	0.029
			1.756	1.275	—		0.024	0.027	0.026
Sample	7	0.426	0.536	0.439	—	0.050	0.045	0.041	0.044
			0.573	0.477	—		0.044	0.048	0.046
			0.613	0.439	—		0.044	0.042	0.047
Sample	8	1.784	—	—	—	0.051	0.049	0.041	0.041
			—	—	—		0.044	0.044	0.045
			—	—	—		0.044	0.048	—
Sample	9	over	over	over	over	0.028	0.029	0.025	—
			over	over	over		0.030	0.028	—
			over	over	over		0.028	0.027	—
Sample	10	2.506	2.846	2.553	—	0.031	0.032	0.029	—
			2.546	2.259	—		0.028	0.028	0.035
			2.549	2.420	—		0.033	0.034	0.026

upper: 3°C, middle: 20°C with desiccants, lower: 20°C
—: not tested
over: overflowed absorbance value

Therefore, prevention of HBV carrier state will lead to the prevention of chronic liver disease. Numerous studies have been done in developing countries to clarify the course of transmission to provide enough data for the anti-HBV program. Previous studies have shown that most of HBV infection occurs in early age in endemic areas. Maternal-child transmission has been proven to be an important cause of HB carrier state in some areas like Singapore (Chan *et al.*, 1985), Taiwan (Stevens *et al.*, 1975) and Japan (Shiraki *et al.*, 1977), but not in other areas (Johnson *et al.*, 1980; Prince *et al.*, 1981; Nasidi *et al.*, 1986; Milne *et al.*, 1987), where horizontal transmission among children are considered to be rather important. To develop an appropriate anti-HB program, including HB vaccination, the large-scale surveillance of age-specific prevalence of HBV markers are needed. Our method seems to be suitable for such a kind of surveillance because of its logistic, economical and technical merits.

But there remain some problems that must be discussed in detail. Werner *et al.* (1985) mentioned that the filter paper method might not be able to detect one-third of the HBsAg positive persons. In our study we used high titer HBsAg involving blood, all of which were over 30 as a cut off index value by RIA. Therefore further studies are necessary regarding low titer HBsAg involving blood and about the quantitativity of this method.

Regarding the dilution rate, values varied from the same to twice as much as the theoretical value. Some possibilities should be considered as reasons:

1. Problem of filter paper
   Blood volume contained in absorbing area might not be constant.
2. Influence of other components of blood or filter paper
3. Problem of elution
   Elution period, temperature and other environmental factors might influence the constancy of elution.

Further examination is needed to clarify the influences of such factors in order to make sure of the reliability of the filter paper method.

Although there are some problems to be solved, the filter paper method will be appropriate for the seroepidemiological field study, especially in developing countries.

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濾紙使用による微量血中のHBsAgの ELISAによる測定

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濾紙法を用いてHBsAgの検出を，ELISAによって行うことを検討したので報告する。

RIA法によりHBsAg陽性を確認した10名と，同様に陰性を確認した10名の計20名について採血用濾紙（東洋濾紙・ストリップⅠ型）に血液の微量を吸着させ，自然乾燥後一定量を切り取り，PBS（pH 7.2）中で抽出（37°C・3 hr）したもの0.1m*l*を検体としてELISA（イムニスHBsAgEIA・三光純薬）によりHBsAgの検出を行った。

RIA法およびELISA原法との比較では，上記の被検者より得た陽性検体200枚，陰性検体100枚を本法により分析した結果と，RIA法の結果は全例で一致した。また，血清0.1m*l*を用いたELISA原法とも全例で一致した。採血用濾紙Ⅰ型に採血した試料を1m*l*のPBSで抽出すると理論上希釈率は0.04となる。本研究では原血清について検量線を作成し，それを基に希釈率を求めたが，その値は0.041-0.082であった。1ケースより10枚の濾紙血を得，本法に於ける測定値の変動を調べた。吸光度がoverflowしなかった6ケースでみると，変動係数は7.4%-14.9%で高い再現性が得られた。採血した濾紙を，①室温（20°C），②室温＋乾燥剤，③冷蔵庫内（3°C）の3条件下で保存し7，14，21日後にそれぞれ測定を行った。陽性例ではいずれの条件下でも，21日目まで吸光度の明らかな低下は認められなかった。また，陰性検体が陽性になることもなかった。

以上により本法は熱帯地域におけるHBsAgの調査に利用可能であると考えられる。

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DIAGNOSIS OF *PNEUMOCYSTIS CARINII* IN SPUTUM LIQUEFIED WITH THE MUCOLYTIC AGENT DITHIOTHREITOL (SPUTASOL)

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Abstract: 1. Light microscopic investigations: Sputasol treatment did not affect the staining properties of either the cyst wall or the parentheses-like structures by Gomori's methenamine silver nitrate (GMS) stain. Detection of *Pneumocystis carinii* was easier in the Sputasol-treated preparations because it was possible to prepare thin smears of the sediment, and because the cysts were concentrated. The amount of time needed for microscopic examination was reduced to the decreased amount of sediment in the Sputasol-treated samples. 2. Transmission electron microscope investigations: In the ultrastructure of *P. carinii* cysts stained with GMS stain, the heavier deposition of silver particles on the internally thickened parts of the cyst wall corresponded exactly with the distinctive appearance of the parentheses-like structures seen in light microscopic observations.

INTRODUCTION

Antemortem diagnosis of *Pneumocystis carinii* pneumonia is based on detecting properly stained cysts of the parasite in sputum, tracheal aspirate, bronchoalveolar lavage (BAL), transbronchial lung biopsy, open lung biopsy, or lung aspirate. In the examination of sputum, cyst concentration is very important. In a previous study (Yoshida *et al.*, 1978), we attempted to achieve cyst concentration by treating sputum with acetyl-L-cysteine. Our initial results were promising and encouraged this follow-up study, in which we evaluated the usefulness of dithiothreitol (Sputasol) treatment of sputum and infected lung tissue in the diagnosis of *P. carinii*. Sputasol is a protective reagent for SH groups (Cleland, 1964), and is widely used for the liquefaction of sputum samples received for bacteriologic examinations (Hammerschlag *et al.*, 1980).

MATERIALS AND METHODS

Specimens: Sputum and BAL were obtained from patients who had received renal transplants; and frozen human lung taken at autopsy of a 23-year-old woman renal transplant recipient.

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Sputasol: Vials of Sputasol powder were obtained from Oxoid Limited, Basingstoke, Hampshire, England, and were prepared as follows: To one vial of Sputasol was added 5 m*l* of sterile distilled water, and the contents mixed gently until the powder was completely dissolved. To this was added aseptically an additional 95 m*l* of sterile water. This solution was used immediately, or stored at 4°C for up to 48 hr. The chemical contents of each vial of Sputasol are listed in the Table 1.

Sample preparation: One volume of sputum or BAL was added to five volumes of Sputasol. This mixture was placed in a 37°C incubator or water bath shaker for 5 min. Following incubation, the samples were filtered through one layer of cotton gauze, then centrifuged at 3,000 rpm for 10 min. Smears of the sediment were prepared, air-dried, fixed in absolute methyl alcohol for 5 min, then stained by Gomori's methenamine silver (GMS) method (Grocott, 1955). The stained smears were examined under both the high dry (400×) and oil immersion (1,000×) lenses of a compound microscope.

Transmission electron microscopy: Small pieces of lung were minced with scissors, rinsed and incubated in Sputasol in a 37°C water bath for 10 min, filtered through cotton gauze, then centrifuged at 3,000 rpm for 10 min. The sediment was fixed in 2.5% glutaraldehyde for 2 hr, then cut into 1 mm^3 blocks. These small blocks were stained with GMS, post-fixed in 1% osmium tetraoxide for 1 hr, dehydrated in a graded series of acetone, and embedded in epoxy resin. Ultrathin sections, obtained with a Porter Blum MT-1 ultramicrotome and glass knife, were stained with uranyl acetate and lead citrate and examined with a JEM 100S electron microscope.

Results

Light microscopy: The results can be summarized as follows: 1. Detection of *P. carinii* was easier in the Sputasol-treated preparations because the cysts were concentrated. 2. Sputasol treatment did not affect the staining properties of either the cyst wall or the parentheses-like structures (Fig. 1). 3. The amount of time needed for microscopic examination was reduced due to the lesser amount of sediment in the Sputasol-treated samples.

Transmission electron microscopy: Figure 2 shows the ultrastructure of a *P. carinii* cyst stained only with GMS. The heavier deposition of silver particles in the internally thickened parts of the cyst wall (arrows) is clearly visible. Figure 3 shows the ultrastructure of a *P. carinii* cyst stained with GMS, then with uranyl acetate and lead citrate. The silver particles are specifically deposited on the electron-lucent middle layer and the thickened portion of the cyst wall (arrow).

Table 1 Vial contents of Sputasol

Dithiothreitol	0.1	g
Sodium chloride	0.78	g
Potassium chloride	0.02	g
Disodium hydrogen phosphate	0.112	g
Potassium dihydrogen phosphate	0.02	g

pH 7.4±0.2

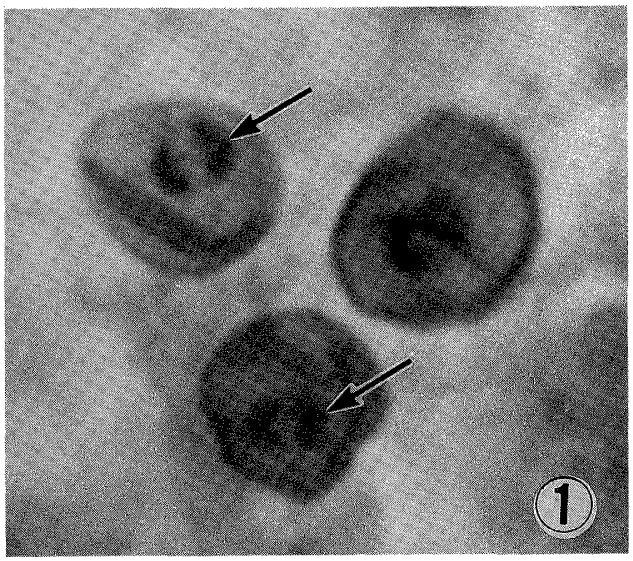

Figure 1 *P. carinii* cysts in sputum liquefied with Sputasol. The cyst wall and the parentheses-like structures (arrows) are clearly visible. (Gomori's methenamine silver nitrate, × 5,000)

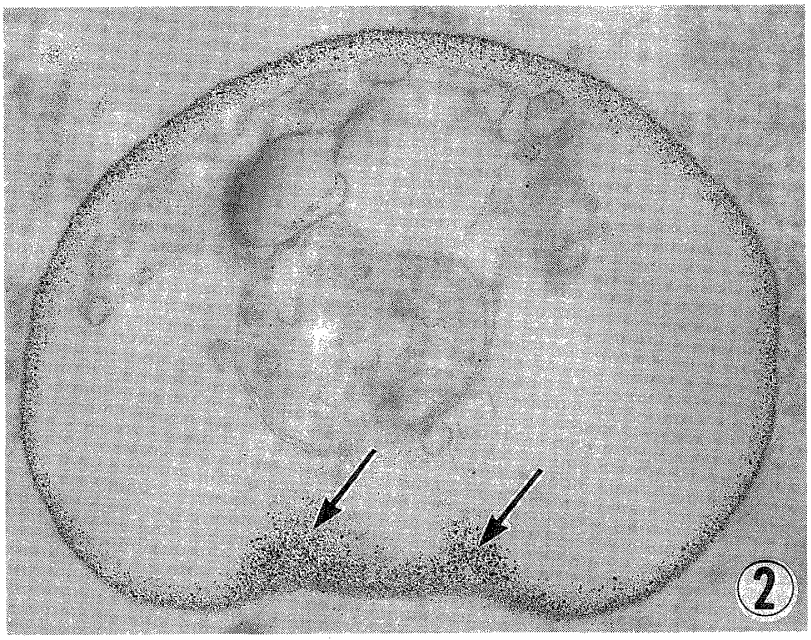

Figure 2 *P. carinii* cyst in lung tissue treated with Sputasol and stained only with Gomori's stain. Note the heavy deposition of silver particles on the internally thickened parts of the cyst wall (arrows). (×25,000)

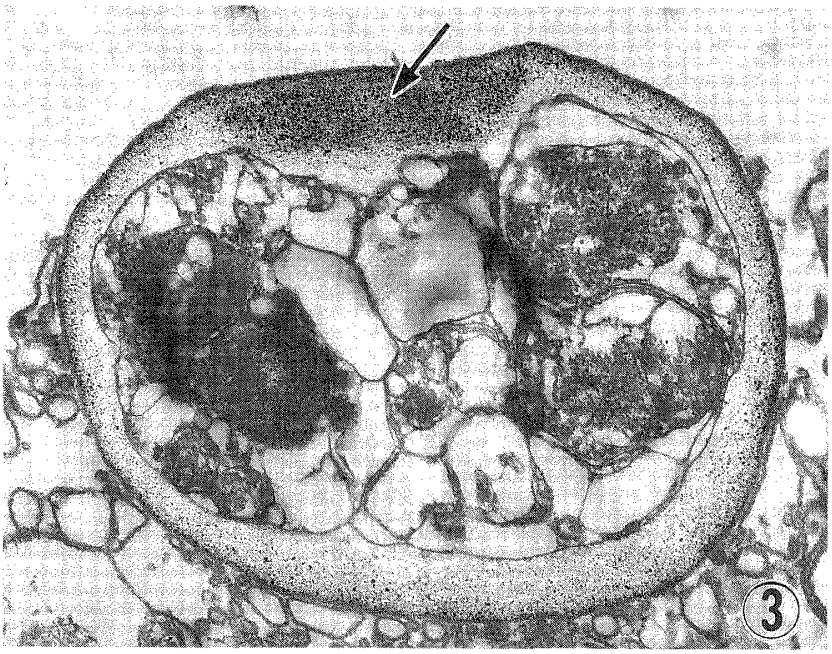

Figure 3 *P. carinii* cyst in lung tissue treated with Sputasol and stained with Gomori's stain and with uranyl acetate and lead citrate.
The silver particles are specifically deposited on the electron-lucent middle layer and on the thickened portion of the cyst wall (arrow). (×25,000)

Discussion

The results indicate that cyst concentration in patient specimens is feasible and that good staining of both the cyst wall and the parentheses-like structures can be obtained following Sputasol treatment. The great benefit of this method in the ease with which cysts in the sputum can be concentrated and detected. In the transmission electron microscope investigations on cysts stained with GMS, the heaviest deposition of silver particles was seen to occur on thickened portions of the cyst wall, areas that correspond exactly with the parentheses-like structures seen in light microscopic observations (Shiota, 1987; Yoshikawa and Yoshida, 1987).

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喀痰液化剤・dithiothreitol (Sputasol) 処理による *Pneumocystis carinii* の診断

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Pneumocystis carinii (Pc) 肺炎の患者の喀痰，および気管支肺胞洗浄液 (BAL) と，Pc 肺炎で死亡した患者の肺を，喀痰液化剤・dithiothreitol (Sputasol・Oxoid) で処理した後，Gomori's methenamine silver nitrate (GMS) 染色を施し，無処理のコントロールと比較検討した。方法：①喀痰の5倍量の Sputasol を加え，37°Cの水浴中あるいは孵卵器内で10分間振盪して液化。BAL では直接3,000 rpm，10分間遠沈後多量の粘液性沈渣がある場合，これの5倍量の Sputasol を加え喀痰と同様の操作を行う。肺は細切後ガーゼ濾過し，充分量の Sputasol を加える。②3,000 rpm10分間遠沈。③沈渣をピペットでよく混和した後スライドグラスに塗抹し，風乾後 GMS 染色を施して400倍と油浸下で観察。また，肺の沈渣はグルタールアルデヒドで固定後，GMS 染色を行い，オスミウムで後固定，ウラニル・鉛で重染し，TEM で観察。結果：嚢子壁および Pc に特異的に認められる括弧状構造物の染色性はコントロールに比べ差異はなかった。コントロールの喀痰および BAL の沈渣塗抹標本では，厚い部位の Pc の染色性は弱く，同一標本上で染色性のばらつきを認めたが，Sputasol 処理標本では均一な厚さに塗抹されており，ばらつきは少なく Pc を検出し易かった。Sputasol 処理の沈渣塗抹量は，コントロールの直接塗抹量に比べ非常に少量になるため，標本枚数が少数で済み，鏡検時間が短縮された。GMS 染色を施した Pc 嚢子の TEM 像では，嚢子壁の内部への肥厚部に多くの銀粒子の沈着が認められ，この肥厚部が光顕で観察される括弧状構造物と一致するものと思われる。以上の結果から本法は Pc 肺炎患者の喀痰，気管吸引物，BAL 等の材料から Pc を検出する場合，集シスト法として操作が比較的簡単で良い方法と考えられる。

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Special lecture

COOPERATIVE MEDICAL RESEARCH PROJECTS IN KENYA

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Introduction

Many developing countries now recognise research to be an important tool for social and economic development. Despite this realisation nearly all of these countries still allocate less than 0.5% of their GNP on research and development (R & D) activities.

In the past 10 years Kenya has been investing increasingly in R & D efforts. To this end, apart from established Universities, Kenya in 1979 established research institutes in the fields of agriculture, health, industry and fisheries. Medical research as a means of improving health care delivery has received considerable attention. In 1987 4.70 million pounds was allocated to medical research compared to 0.61 million pounds in 1980.

The above allocations, however, are far from adequate to satisfy medical research needs. It is with this realisation that the Government welcomes increasing international and regional cooperation in medical research.

Relevant Statistics

Kenya has a population of 22 million of which 85% is rural and 15% urban. The population is young with 51% being 15 years and under. The mean infant mortality rate (IMR) now stands at less than 90 per 1,000 births (range 45-220).

The five major causes of morbidity in order of importance are:

Malaria 23.6%, ARI 20.2%, Skin diseases 6.5%, Diarrhoea 5.8% and Intestinal worms 5.5%.

Of the parasitic diseases the most important in terms of morbidity and mortality are malaria and schistosomiasis.

Health Research

Most of the medical research activities in Kenya are located at the Kenya Medical Research Institute (KEMRI). KEMRI has its headquarters located in Nairobi. Peripheral centres exist in Kisumu, Busia and Mombasa. The establishment of the headquarters and central laboratory complex of KEMRI was generously funded by the Japanese Government to the tune of approximately $10 million. This capital development, as we shall see later, is also supported through technical cooperation through JICA.

The major research programmes of KEMRI are:

Malaria, Schistosomiasis, Leishmaniasis, Diarrhoea disease, ARI, Viral diseases, STD and Traditional medicines.

Since, as was stated earlier, local resources are inadequate to meet research needs KEMRI has entered into cooperative research agreements with selected Governments and agencies. The major ones of these are:

JICA-parasitology, bacteriology, virology
WRAIR (USA)—malaria, leishmaniasis
CDC (USA)—malaria
WHO—TDR diseases, STD, CDD
IDRC—dental health, primary health care

By far the largest input in terms of resource allocation and manpower development to KEMRI comes from JICA.

Kenya-Japan Cooperation

The Japanese have had interest in health related research in Kenya for many years. However serious technical cooperation in this field started in 1979 with the establishment of the Communicable Disease Research and Control Project (CDRCP). This 5 year project which terminated in 1984 identified diarrhoeal disease and schistosomiasis as the main areas of mutual research interest. The participating Japanese institution was Nagasaki University.

In 1985 a further 5 years technical cooperation agreement was negotiated. This agreement identified the following fields for joint research and control efforts:—

Parasitology: schistosomiasis
Bacteriology: diarrhoeal diseases
Virology: diarrhoeal disease, viral hepatitis

The participating institutions were expanded to include Tokai University, Sapporo and Iwate Medical Universities, in addition to Nagasaki University.

The cooperation comprises actual research, provision of research materials, training of Kenyans in Japan and deployment of Japanese experts to Kenya.

Achievements

a) Training:

So far over 15 Kenyans have trained in Japan in such disciplines as parasitology, tropical medicine, electron microscopy, medical engineering, bacteriology, virology, etc.

b) Research:

Parasitology: Schistosomiasis affects over 2 million Kenyans. In certain areas over 90% of the children are infected with either *S. mansoni* or *S. haematobium*. The present research project in Kwale has looked at prevalence, diagnostic methods, cercariometrics, malacology and intervention.

So far, safe water has been provided in a pilot area and treatment with praziquantel together with health education have been offered. Intensity of infection has been reduced by 80%. Community awareness has increased.

Bacteriology: The project has focussed on studies of intestinal flora, bacterial ecology and enteric infections. So far intensive analysis of 15 healthy and diarrhoeic children has been done. These studies complement those of viral aetiology of diarrhoea.

Virology: Rota viruses have been shown to be significant causes of infant diarrhoea. The Kenyan study has attempted to determine the role of rota-virus in childhood gastroenteritis. Studies have also attempted to examine the serotypes involved and factors responsible for susceptibility.

A cohort of 100 children is being followed up and screening for rota-virus using ELISA and electron microscopy is done frequently.

c) Logistics:
Equipment, supplies, transport and travel costs are provided. This greatly helps in the smooth implementation of the project.

d) Experts:
These have come and stayed for periods of 3-12 months and some even longer. They have been instrumental in the initiation and continuation of projects and for training within projects.

The future

Japan has assisted Kenya in many technical fields such as forestry, agriculture, mining, energy, roads only to mention a few. In the field of health research, which is the subject of this meeting, Japan has closely worked with Kenya to develop research and technical capacity. At the same time Kenya has provided the opportunities for Japanese medical scientists to improve their skills in Tropical Medicine. The exploitation of these opportunities should be encouraged. The future is encouraging and it is my hope that more Japanese resources can be put to this worthy effort.

Symposium
I Tropical Infectious Diseases and Drugs

1 INSECTICIDES

1a INSECTICIDES FOR USE IN JAPANESE ENCEPHALITIS CONTROL PROGRAMMES

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The number of Japanese encephalitis (JE) human cases in Japan greatly changed in years. Although hundreds or more than 2,000 cases appeared in a year in 1960's, the number remarkably decreased after 1972 and less than one hundred cases only were reported every year. It is very likely that a most important factor responsible for this reduction is the decreased density of the vector mosquito, *Culex tritaeniorhynchus*, and the decrease in the vector density is due to the switch from chlorinated to organophosphorus and carbamate agricultural insecticides sprayed in the rice field, which is the main breeding place of the vector mosquito. The slight increase of the vector in 1980's seems to be due to the development of resistance to the insecticides. Thus the population of the vector and therefore the epidemic of JE also are influenced by the application of agricultural insecticides in the field.

There are many insecticides that have been proven to be effective for the control of the vector mosquito in the laboratory and in the field. The application of insecticides to rice fields is effective in controlling the vector population, but usually only for one or two weeks. Therefore, if we wish to control vectors by larviciding, several applications will be necessary in a year. This means that in view of a vast area of rice fields, the application of larvicides for the control of JE will be extremely costly and probably of no operational value in usual circumstances. Moreover, the chemical control by using organophosphorus and carbamate insecticides became completely ineffective in recent years due to the development of insecticide resistance. The aerial spray of insecticides against the adult population of the vector does not seem to be of practical value for the same reason, since the adult mosquitoes rest in very large areas of rice fields and nearby grassy lands.

To prevent transmission of JE, the first measure to consider is vaccinating humans because of its effectiveness. However, it is not easy to raise the rate of persons receiving vaccination. The vaccination of pigs, which are the most important amplifying animal of JE virus, is usually of little practical value in view of the cost involved. Separating pigs from rice fields is the surest way to reduce pig-mosquito contact, but there is a limitation to its realization.

As mentioned in the above, most measures to control vectors by insecticides are of no operational value at least in the present situation, however effective they are, and it is not easy to further decrease the number of cases by measures to humans and pigs. A promising approach would be to reduce the number of vector mosquitoes engorged with pig blood, which

may include mosquitoes just infected with JE virus, by using, for example, insecticide impregnating netting at pigsties.

1b INSECTICIDES FOR USE IN DENGUE CONTROL PROGRAMMES

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Aedes aegypti, the main vector of dengue fever, breeds in various kinds of containers in and around the human dwelling, for example, ant-traps, earthen jars, flower vases and tin cans. As most of these breeding sites are artificial, it can be said that the epidemic of dengue fever is man-made. Only vector control are most effective measure to control dengue fever at the present time, because the vaccine against dengue viruses has not been developed for practical application.

There are two measures against *Ae. aegypti* as the vector of dengue fever. The first one is keeping the population of *Ae. aegypti* constantly in low density below the threshold transmission level in a certain area, in order to prevent the outbreak of dengue fever. The environmental control around houses by inhabitants themselves who receives health education is most recommended to control *Ae. aegypti.* For example, removing empty tin cans, bottles and rubber tires from their back yard, changing the water in flower vases frequently, putting salt into ant-traps and covering earthen jars and tunks holding drinking water would be easy, economical and effective methods. However, human behaviour or inhabitants' habit may impede to dry up all disused water and put the lid on water jars completely in some areas, where we are compelled to apply the larvicide to those water containers. Many kinds of larvicide (organophosphate, IGR) may be effective to *Ae. aegypti* larvae as shown by laboratory experiment. Because most of these containers are used for holding drinking water, we must take into consideration that the larvicide is not toxic to human and other vertebrate animals and has not a strange taste or smell.

The second measure is to suppressing the epidemic of dengue fever instantly in a certain area. If the transmission of dengue viruses is prevented by the control of the adult population of *Ae. aegypti* for 14 to 20 days, the epidemic will be terminated from the area. Fogging of insecticides (fenitrothion, permethrin and others) to houses, where this mosquito rests and feeds on human blood, is used for this purpose. Recently ULV is recommended to control adult *Ae. aegypti,* because it is more effective to adults and also kills larvae in containers.

As larvae of *Ae. albopictus* occupies small containers around houses, the basic method to control this mosquito is the same as that for *Ae. aegypti* mentioned above.

1c INSECTICIDES FOR USE IN ANTIMALARIA PROGRAMMES

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Indoor residual spraying of DDT was a major strategy of the anti-malaria project during the 1950s and 1960s in various parts of the world. The effects were dramatic. It reduced malaria incidence quickly and eliminated endemic areas in many countries. A malignant malaria in the Yaeyama Islands of Japan which had long bothered residents was also eradicated using this method. DDT spraying, however, experienced a crisis in the 1970s because of environmental pollution and the health hazard. In addition, inhabitants of endemic areas often refused the spraying because it stained the interior of their dwellings. The spray operation was also curtailed by financial difficulties. The cost of malaria control operations has risen greatly due to increasing costs of transport, fuel and personnel. A shortage of funds for the purchase and distribution of chemicals by national health services has resulted in an inadequate control operation. New chemicals are now being tested and adopted in various areas, and new application methodologies, such as selective spraying of houses and cattle sheds, based on the resting preferences of vectors are being used in an effort to reduce costs.

Alternatives are now emerging with the involvement of communities in anti-vector measures. A mosquito-net or bed-net is a potentially excellent anti-vector means, but it may soon be torn. Therefore, impregnation of the net with an insecticide such as a synthetic pyrethroid has been adopted to improve the protection efficiency. A wide-mesh net curtain, which does not obstruct the ventilation of the room and even allows mosquitoes to pass through easily, can be treated with an insecticde and may be more acceptable to occupants in hot and humid areas. In a trial in a Toyama Prefecture pigsty, the total number of mosquitoes collected in a light trap surrounded by a net impregnated with phenothrin was much less than half the number using an untreated net. The treated net reduced the proportion of surviving mosquitoes to a negligible level and greatly reduced the per cent of fed mosquitoes. There is a need for further study in both the laboratory and field for accurate assessment of the effect of insecticides not only to kill but also on vector mosquito response. A series of laboratory tests comparing different chemicals was carried out to select an insecticide suitable for net treatment. Results revealed that nets impregnated with pyrethroids such as phenothrin and cyphenothrin reduced both the number of mosquitoes passing through and their feeding activity.

2 ANTI-PARASITIC DRUGS

2a THE ANTI-PARASITIC DRUGS IN CURRENT USE

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Parasitic diseases which were rampant during and just after World War II have rapidly subsided these days thanks to exhaustive disease control measures, improved environmental sanitation and a change of eating habits.

On the other hand, however, as international exchange has lately become more and more frequent such clinically important issues as how to control imported parasitic diseases, parasitic diseases set off due to immuno-deficiency state and those common to both men and animals have given rise to public attention.

Meanwhile anti-parasitic drugs are difficult to obtain these days because parasitic diseases are scarce now in Japan and, in addition, some of the drugs have troublesome side effects.

In order to counter these problems we instituted in 1988 a task force for studing drugs for the imported parasitic diseases and have had 15 items of difficult-to-obtain drugs in our charge for "experimental purpose", which have been dispensed on request.

Results:

1) The drugs had been distributed among patients for 7 years from 1980 through 1987 on 1,101 occasions (about 150-200 cases annually). Malaria (287 cases) topped the disease list, followed by strongyloidiasis (210 cases), trichocephaliasis (193 cases), clonorchiasis (97 cases), amebic dysentery (50 cases) and pneumocystis carinii pneumonia (50 cases), etc.

Anti-malarial drugs of primaquine, chloroquine, fansidar, etc. had been most requested, then mebendazole for trichocephaliasis, thiabendazole for strongyloidiasis and praziquantel for trematodiasis (clonorchiasis), etc., in that order.

2) Status quo of imported parasitic diseases: There had been 383 patients (287 Japanese and 96 foreigners) with tropical parasitic diseases, 268 of them suffering from malaria, followed by clonorchiasis (32), trichocephaliasis (30), lambliasis (21) and various cestoidiasis cases (7). Such uncommon diseases in Japan as trypanosomiasis (1), Kala azar (1) and bilharziasis (1) were also found among the patients. Most of the presumed areas of original infections with the diseases were east-west Asian and Africal countries. Malaria patients comprised 251 males and 17 females, most of them in their thirties.

Assortments of malaria-originated areas, number of patients and malaria species were: Asian countries (125 patients: 87 *P. vivax,* 16 *P. falciparum,* 2 *P. malariae* and 20 unknown species), African countries (72 patients: 27 *P. vivax,* 27 *P. falciparum,* 1 *P. malariae* and 17 unknown species) and Oceanian countries (22 patients: 13 *P. vivax* and 9 *P. falciparum*).

The purposes of the patients' travel were sightseeing, business and academic investigation, in that order, for Japanese citizens, meanwhile they were training, business, sightseeing and entry into Japan as refugees for foreigners.

The task force for development and procurement of drugs against imported parasitic diseases has made every effort since 1980 to solve aforementioned problems and, as a result, some drugs have come into the market.

The above-described facts are the status quo of the imported parasitic diseases in Japan.

2b NEUROPHARMACOLOGICAL APPROACH TO ANTHELMINTIC DRUGS

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Chemotherapy is essentially important in treating patients with helminthic parasites. Since treatments making host resistant to the infections are generally not promising in helminthiasis, the significance of the chemotherapy in controlling the diseases should also be emphasized. A basic approach to the chemotherapy against helminthiasis is presented in this report.

Why did we study anthelmintic drugs neuropharmacologically ?

Traditionally, many chemotherapeutic studies against helminthiasis have been aimed at energy metabolism of the worms: i. e., effects of anthelmintics on the metabolism and mechanism of the metabolism. Although the mechanism of the energy metabolism is basically common in nemathelminthes and platyhelminthes, there is a marked difference in sensitivity to anthelmintics between these two groups of helminths. To reveal mechanisms of the difference, neuropharmacological mechanism was taken up as another target for chemotherapy. Motility of worms was regarded as the most useful parameter and an isotonic transducer method was introduced to detect the faint motility of smaller species *in vitro*. *Angiostrongylus cantonensis* and *Hymenolepis diminuta* were selected for representative models of nemathelminthes and platyhelminthes, respectively. Thus, effects of various neuropharmacological drugs on the motility of helminths including the model worms were studied by the isotonic transducer method. It was suggested from the findings reported that acetylcholine (ACh) and γ-aminobutyric acid (GABA) in nematodes and 5-hydroxytryptamine (5-HT, serotonin) and ACh in platyheminthes act as stimulatory and inhibitory neurotransmitters, respectively. It is likely that the difference in the sensitivity to anthelmintics between nemathelminthes and platyhelminthes is attributable to the difference in the neuropharmacological mechanism. Then, we studied effects of various anthelmintics in this aspect. Antinematodal anthelmintics, for example, were classified into three groups; anthelmintics including pyrantel act on cholinergic mechanism, ones such as piperazine and milbemycin D act on gabergic mechanism, and ones like diethylcarbamazine affect both mechanisms. Two topics on neuropharmacological actions of these anthelmintics are presented as follows.

Why is pyrantel used clinically only against intestinal nematodes ?

Pyrantel at 10^{-5} M or less affected the motility of nematodes and isolated host tissues, while no effect was seen in cestodes and trematodes. For the selectivity of the action of pyrantel on nemathelminthes and platyhelminthes, it is assumed that pyrantel acts on nematodes through stimulating nicotinic ACh receptors in them, and that the anthelmintic doesn't affect platyhelminthes since they lack the nicotinic ACh receptors. In spite of the effects on the nicotinic receptors in host tissues *in vitro*, pyrantel doesn't affect host adversely, because the drug is hardly absorbed through the intestinal tract of the host and also because nematodes are affected at lower concentrations than host tissues.

How are complicated actions of an anthelmintic elicited ?

Some anthelmintics such as levamisole are reported to act on some different sites including the energy metabolism and neuropharmacological mechanism of helminths. On the other hand, it is reported in the case of piperazine that a secondary inhibition in energy metabolism is derived from a primary stimulation on the gabergic mechanism in *Ascaris*. As another example of the latter cases, effects of milbemycin D on *A. cantonensis* were shown. Milbemycin D paralyzed the worm *in vitro* at 10^{-11} g/m*l* or more, probably through gabergic stimulation. A single oral dose, 5 mg/kg of the drug paralyzed the worm in rats, and the effect lasted about 1 week. When infected rats were treated with 9 doses of 5 mg/kg/week, the paralysis seemed secondarily to cause the worms inhibition in food intake, energy metabolism and reproductive function. No first stage larvae were seen in host feces persistently after the second dose, and no food materials in the intestinal tract of the worms and no eggs and first stage larvae in the lung tissues of the host were observed at sacrifice after the final dose. Thus, as shown in the effects of milbemycin D, many complicated effects of anthelmintics seem to be attributable to the fact that various mechanisms in the worms are closely related each other.

Why is chemotherapy against helminthiasis with tissue helminths difficult ?

Finally, anthelmintics against intestinal and tissue helminths were compared. Intestinal helminths can be rather easily treated with anthelmintics, because there are some advantageous aspects which make chemotherapy easier. For example, even a reversible action is effective because of the peristalsis of the host intestine by which paralyzed worms can be expelled, and even toxic drugs like pyrantel can be useful unless absorbed through the intestinal tract of host. Thus, we have had effective anthelmintics against almost intestinal helminths and will obtain more useful ones in respects of efficacy, safety and spectrum. On the other hand, there remains extremely difficult situation regarding tissue helminths. It is hard to get more effective and less toxic anthelmintics which bear irreversible or long-lasting action when considered their living environments. Even if we could get such anthelmintics and kill the worms successfully, we must be faced with another problem. Adverse systemic effects may be elicited by allergens which are released from the killed worms and distributed through the blood system. This means that many therapeutic treatments are nescessary for effective therapy in addition to killing worms. Thus many difficult problems remain to be solved in the chemotherapy of helminthiasis with tissue helminths.

3 ANTI-MICROBIAL AGENTS AND THEIR DEVELOPMENTAL STATE

3a ENTERIC BACTERIA IN ASIAN COUNTRIES AND THEIR DRUG RESISTANCE

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Antimicrobial agents are commonly used therapeutically and prophylactically for diarrheal diseases. Resistance of enteropathogens to these antimicrobials may prevent the effect of such therapy, and thus information on distribution of antimicrobial-resistant enteropathogens are indispensable therapeutically. The information may also lead to understand epidemiology of diarrheal diseases.

In this paper we describe enteric bacteria causing diarrheal diseases, especially travelers' diarrhea, in Asian countries, and antimicrobial resistance of the enteropathogens detected from the travelers.

Enteric bacteria in travelers' diarrhea

Our surveys on travelers returning from abroad mainly from Asian countries during the last decade (1978-1987) showed that approximately 60% of the diarrheal cases from abroad were possibly due to bacterial infections. The most common causal agent was enterotoxigenic *Escherichia coli* (ETEC) occupying 47.2% of the total isolates. It was followed in order by *Salmonella* (12.8%), *Vibrio parahaemolyticus* (10.9%), *Shigella* spp. (8.0%) and *Campylobacter jejuni/coli* (7.4%). *Vibrio cholerae* O1 was detected at a rate of 0.5%. When detection frequency of each enteropathogen was compared with countries where the infection occurred, some geographical tendency were observed among Asian countries. Namely, infection due to ETEC was common in most Asian countries, whereas *V. parahaemolyticus* infection was more common in Eastern and Southeastern Asia than Western Asia. *Shigella* and *Campylobacter* infections were prevalent in West Asia, but not so common in other Asian countries. It is also notable that infections with multiply pathogens are very common in travelers' diarrheas.

Antimicrobial resistance of enteric bacteria

Antimicrobial susceptibility data shown here were mainly derived from our surveys on travelers' diarrhea or through data exchange project of Southeast Asian Medical Information Center (SEAMIC). Antimicrobial agents tested included chroramphenicol (C), tetracycline (T), streptomycin (S), kanamycin (K), ampicillin (A) and sulfamethoxazole-trimethoprim (X).

In *Shigella*, frequency of resistant strains (mostly multiple drug resistance) was the highest among enteropathogens tested, but no marked geographical difference in incidence was observed in incidence between Japan and other Asian countries. Among antimicrobials tested, resistance to S, T, and C was remarkably high, followed by A and X, but K resistance remained still relatively low. Frequency of resistance of non-typhoidal *Salmonella* was about 20% both in Japan and in other Asian countries, but resistance to each drug varied by countries and tended to increase gradually year by year. It is also noteworthy that *Salmonella* resitance is markedly different by serovar. High resistant serovars found in Japan were Hadar, Litchfield, Muenchen, Typhimurium and Blockley, whereas in other Asian countries were Blockley, Hadar, Typhimurium, Krefeld and Panama. In *Salmonella* ser. Typhi, incidence of resistant strain was still quite low, but it should be noted that there were resistant strains which were mediated by transferrable R plasmid in Asian countries such as Indonesia and Thailand. Of interest, ser. Typhi R plasmids found in Asian countries belonged to incompatibility group H_1 and appeared to be identical to that in Mexican outbreak in 1970s. Resistance of diarrheagenic *E. coli* was about 50%, and no remarkable differences were observed in diarrheagenic types of organisms (ETEC, EIEC or EPEC) or in places of isolation. Diarrheagenic vibrios remained largely susceptible against antimicrobials tested.

However, recently resistant *V. cholerae* O1 strains were found to distribute in Thailand and Philippines, and most of them were mediated by transfferable R plasmid of incompatibility group C.

3b DRUG RESISTANCE OF *VIBRIO CHOLERAE* O1 ISOLATED IN TROPICAL AREA

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There have been frequent epidemics of cholera in Africa since the 7th cholera pandemic that originated from Celebes Island in Indonesia in 1961, and reached the African continent in 1970.

Since then, cholera is endemic in these areas and sometimes becomes epidemic. Antibiotics are given routinely to patients with cholera because it decreases the duration and volume of purging, the duration of excretion of vibrio and the amount of fluid replacement required, although fluid replacement is the most important component of therapy. Drug resistant strains of *Vibrio cholerae* has been isolated sporadically in endemic areas.

However, no extensive emergence of multiply drug-resistant strains which becomes a serious problem clinically, has occurred.

However, In 1979, Mhalu *et al.* reported that the strains of *V. cholerae* resistant to tetracycline (TC), a drug considered to be very effective against cholera, were also resistant to streptomycin (SM) and ampicillin (ABPC), were isolated in Tanzania. All isolates during the first month of the outbreak were fully sensitive to TC, but 76% of isolates were resistant to the drugs after five months of extensive use of TC therapeutically and prophylactically. In 1980, Glass *et al.* also reported the emergence of multiply drug-resistant strains of *V. cholerae* in Bangladesh. This paper describes an outbreak of cholera and the emergence of multiply drug-resistant strains of *V. cholerae* in Kenya which we experienced during The Communicable Diseases Research and Control Project between Kenya and Japan supported by the Japan International Cooperation Agency. Two hundred and seventy two strains of *V. cholerae* O1 biotype El Tor were isolated from six cholera outbreak and sporadic cases during 1980 and 1981 by Iwanaga *et al.* dispatched as the first team but no multiple drug-resistant strains of *V. cholerae* were found.

However, multiple drug-resistant strains started to be isolated in 1982 in the South Nyanza District near Lake Victoria and account for 41% of the strains isolated. In April, 1983 when we obtained information about cholera outbreak, we started to make a cholera surveillance in the areas of South Nyanza, especially at Homa Bay District Hospital, Ombo Hospital and other health centers. We isolated and collected 244 strains of *V. cholerae* from inpatients, high school students, prisoners and healthy carriers. Out of 244, 235 strains of *V. cholerae* were Ogawa type, nine were Inaba type. All the isolates were El Tor biotype except one untypable and Celebes original type in prophage typing. All the isolates were sensitive to nalidixic acid and chloramphenicol and 183 out of 244 strains were resistant to TC, SM, ABPC and ST compound. A cholera outbreak occurred in Nairobi on November in 1983 and

in Kisum in March, 1984. The number of strains isolated in Nairobi and Kisum was 70 and 23, respectively. All the strains isolated were biotype El Tor, Celebes original in prophage typing. The number of resistant strains to three drugs (TC, SM, ABPC) were 64 (91%) and 18 (78%), respectively. The resistance of these strains was transmissible. According to Morris *et al.*, all Kenyan resistant strains contain a group C incompatibility plasmid of ca 100 MD and which is different from those in Tanzanian and Bangladesh strains in restriction endonuclease digest pattern. Therefore, the multiple drug-resistant strain in Kenya was probably not directly brought from the Tanzania border, although it occurred near the border. In fact, close contacts of cholera patients started to be administered tetracycline by the cholera surveillance team for prophylactic use in 1982 and the number of multiple drug-resistant strains was increasing in this year. An extensive chemotherapy is thought to be closely associated with the development of drug resistance. Furthermore, inadequate management for cholera, for example, insufficiency of dosage of drug, failure of treatment and drop-out cases during the treatment of cholera might have resulted in the wide spreading of cholera and development of drug resistance. Cholera outbreaks due to multiple drug-resistant strains were also seen in Somalia and India.

3c CURRENT STATUS OF RESPIRATORY PATHOGENS AND DRUG RESISTANCE

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Infectious diseases are still very common in the tropics and mortality rate due to pulmonary infections has been the highest among infants.

In this paper, the recent situations on bacterial respiratory infections and drug resistance in the area of the southeast Asia based on data mainly from the Research Institute for Tropical Medicine of the Philippines (RITM) and WHO/PRO reports were discussed.

1. Current status of acute respiratory infections in the RITM; About 30% of the inpatient was occupied by pulmonary infections in this hospital and *S. pneumoniae* and *H. influenzae* were the most important causative micro-organisms among the patients with acute pneumonia. On the other hand, more than half cases autopsied had also accompanied lethal pneumonia due to gram-negative bacteria or measles. Although the isolation of *L. pneumophilla* which is well known as a pathogen of nosocomial or opportunistic infection was not yet reported from the clinical specimens of the patients with pneumonia in the Philippines, we succeeded to isolate the strains from the water of cooling towers of hotels and hospitals in Manila with a cooperation of laboratory staff of the RITM. This fact suggested the possibility of existence of the patients with pneumonia due to *Legionella* in this country.

2. Bacterial drug resistance; Resistant strain of *S. pneumoniae* to PCG is reported from the Philippines and Malaysia with the percentages of 2 and 0.2%, respectively, although the frequency rate of resistance to erythromicin or tetracycline was lower compared to that of our country.

None of resistance to PCG was yet reported on *S. pyogenes.*

More than 75% of *S. aureus* produces β-lactamase and frequency of methicillin resistant *S. aureus* (MRSA) varied 0-26% according to countries.

Ampicillin resistant *H. influenzae* was observed with an incidence of 2-20% and most of them produced β-lactamase.

3d PRESENT STATE OF DEVELOPMENT OF ANTIMICROBIAL AGENTS

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The rationales of the development of new antimicrobial agents are (1) progress of medical treatment, (2) change in pathogenic organisms, and (3) increase of medication cost.

The progress of medical treatment has resulted in the prolongation of life of patients, which is accompanied by the increase of patients exposed to the high risk of infectious diseases. For example, the patients treated with anti-cancer drugs on cancers or with immunosuppressive drugs on organ transplantation become susceptible not only to a variety of weakly pathogenic bacteria but also to viruses and fungi. For those immunocompromised patients, antibacterial agents that have broad-spectrum activity and strong bactericidal action are needed. Many of the aged constrained on beds often suffer from the bed-sore that is regarded as the reservoir for methicillin-cephem resistasnt *Staphylococcus aureus* (MRSA). Those factors related to the progress of medical care should be taken into account for the future development of antimicrobial agents.

The change in pathogenic organisms is believed to be related to the usage of antimicrobial agents. The biggest problem in this concern is the outbreak of MRSA, which is said to have some relation to the use of the third-generation cephalosporins having weaker activity than the first- and second-generation ones. Soon after the clinical introduction of new quinolones, the quinolone-resistant bacteria have rapidly increased, especially among *S. aureus, Serratia marcescens,* and *Pseudomonas aeruginosa.* The emergence of resistance to newly introduced antibiotics has been repeatedly experienced. The fight against resistance may continue endlessly.

The increase of medical care cost has become so big that the national budget is threatened by it. In order to reduce the medication cost, every effort is being made. The shortening of the hospitalization period is one of the ways to cope with such a desire. The demand for orally active or suppository drugs is increasing in this context. As to the parenteral antibiotics, long half life or sustained release drugs will be needed in the future.

The major categories of antibacterial agents being persued are (1) cephalosporins, (2) new quinolones, and (3) β-lactams with novel nuclei. The development of aminoglycosides and macrolides is continuing but less actively. The development of penicillines, those active until 1970s, has almost terminated.

The development of cephalosporins is directed toward the expansion of the antibacterial spectrum covering *S. aureus* and/or *P. aeruginosa*, and the orally active compounds. The

development of cephalosporins has proceeded from first-generation (represented by cephalothin and cefazolin), second-generation (cefotiam, cefmetazole), to third-generation (cefotaxime, latamoxef, ceftazidime). During that process, the activity against bacteria of *Enterobacteriaceae* was greatly increased. But, as mentioned before, the activity against gram-positive bacteria was reduced in the third-generation compounds. Flomoxef and cefuzonam are the cephalosporins having improved activity against *S. aureus*. Constant efforts have been made to improve the activity against *P. aeruginosa*; cefpimizole and cefpiramide are recently launched compounds. E-1040, which has potent antipseudomonal activity, is now being developed. Cephalosporins active against both *S. aureus* and *P. aeruginosa* besides against bacteria of *Enterobacteriaceae,* though never launched yet, is the next goal to be attained. HR-810, now under clinical evaluation, is an example of such compound. Increase of the bactericidal activity is another goal. Cefminox shows potent bactericidal activity not only against growing bacteria but also against non-growing ones; β-lactam antibiotics useally kill bacteria only at the growing state.

Orally active cephalosporins have been energetically persued. There are two categories for them: one is the free form and the other ester form. Cefixine is an example of the former, and cefteram pivoxil and cefuroxime axetil, the examples of the latter, that were launched recently. Several orally active cephalosporins are in the late stage of clinical evaluation: ceftibuten (7432-S), FK-482, and Ly-163,892 (KT-3777) as free form, and CS-807, cefotiam hexetil, and BMY-28271 as ester form.

The new fluorinated quinolones have opened up a new era of antibacterial therapy. Norfloxacin was the first to be launched and ofloxacin, enoxacin, and ciprofloxacin were launched subsequently. Nalidixic acid and other older generation quinolones are active mainly against gram-negative bacteria and their clinical application was restricted to urinary tract, otolaryngological, and gastrointestinal tract infections. The introduction of fluorine into the quinolone ring not only improved the activity against gram-negative bacteria, but also expanded the spectrum to gram-positive bacteria. Moreover, the pharmacokinetic properties have also been improved, thereby enabling the new quinolones to be used for respiratory tract and soft tissue infections also. Lemofloxacin (NY-198), Fleroxacin (AM-833), and T-3262 are now under clinical evaluation.

β-Lactams with novel nuclei such as carbapenem, nocardicin, clavulanic acid, and sulfazecin (monobactam) were discovered from microbial cultures in the late 1970s. Of those compounds, clavulanic acid, a β-lactamase-inhibitor, has been clinically in combination with amoxicillin (augmentin). Sulbactam is another β-lactamase-inhibitor utilized. The discovery of sulfazecin is connected to the development of new monobactam antibiotics such as aztreonam and carumonam. They are active selectively against aerobic gram-negative bacteria including *P. aeruginosa.* The first carbapenem antibiotic clinically introduced is imipenem. Since it is susceptible to hydrolysis by the renal dehydrodipeptidase, the combination with cilastatin, a dehydrodipeptidase inhibitor, was necessary, Since carbapenems show broad spectrum and potent bactericidal activity, they seem to be suitable for the treatment of infections in immunocompromised patients, and newer carbapenems such as SM-7338 and CS-976 are now being clinically evaluated.

At the meeting, the comparison of statistics of the use of antibacterial agents in Japan, the United States, Europian countries, and tropical countries, which shows great contrast, was also discussed.

II Present Status and Topics in Development of Vaccines against Tropical Infectious Diseases

1 VACCINES FOR PROTOZOAL DISEASES

1a SEVERAL NOTABLE ACHIEVEMENTS IN RECENT RESEARCH ON MALARIA VACCINE

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Since the start of The Special Programme for Research and Training in Tropical Diseases, studies on malaria vaccine have been one of the greatest concern in modern biomedical science and have attracted attentions and participations of many top scientists toward this field. Sporozoite vaccine has been studied intensively by the group of Department of Molecular Parasitology of New York University headed by Professor Ruth Nussenzweig who started the study as early as in 1967. The sporozoites taken from mosquitoes are mixed with mosquitoe tissues cells, cell debris and contaminants. Difficulties in the preparation of large amount of parasite materials in a purified form hampered further progress in the studies. The problem was solved by the introduction of monoclonal antibody technique followed by genetic engineering. After a successive studies, eventually repetitive peptide (NANP)3 was identified as circumsporozoite epitope which works against the attack by protective antibodies. The repetitive peptide can be a candidate of sporozoite vaccine. Indeed, two independent groups reported that some human volunteers who were vaccinated with (NANP)3 bound tetanus toxoid did not develop malaria after being bitten by malarious mosquitoes. (NANP)3 works as B-cell epitope hence unable to provoke T-cell sensitization. (NANP)3 bound with lysin network makes a mass of peptide, and the mass can elicit T-cell activation. Gamma interferon released from the sensitized T-cell seems to suppress the development of extra-erythrocytic stage, which also work adversely toward parasites. Extraerythrocytic stage of malaria parasite is very difficult to study, because this stage of parasite only occupies really small part of the liver. Isolation and purification of liver stage material is almost impossible. The problem was solved by using polyclonal antibody taken from a peculiar person who had taken chloroquine every day until 27 years old. The serum was reactive to liver stage parasite but not to blood stage parasite. Using this serum, an epitope of liver stage parasite was identified. It still remains to be studied if this epitope works for protection or the epitope is only an antigenic polypeptide which does not induce resistance to the liver stage parasite. Extensive studies have been reported on the epitope from blood stage parasite. Epitope p190 and p155 seem to be promising candidates, however, the peptides need Freunds' complete adjuvant to induce resistance in the experimental monkeys. Still, much studies are needed before test to humans. Synthetic hybrid polymers reported by Pattaroyo *et al.* (a group in Bogoda) presented a promising result in an

experiment on human volunteers in suppressing development of parasitemia after experimental mosquitoe bites. The experiment also supported a new principle in the production of synthetic vaccine by combining different effective epitopes into one substance. The resulted large molecule eventually induced potent immune response in the given individuals, and multi-operative effect may be expected. An attempt to prepare highly immunogenic mutant of *P. berghei* has been worked by us. Such parasite derivative was obtained after giving high dose irradiation to a pool of parasitized erythrocytes. The derived parasite changed the feature and became permanent low virulence organism which, in addition, caused a long lasting immunity in groups of mice. Effective molecule from the obtained parasite are now being studied. A 230 KD molecule which is unique to the derivative parasite was identified.

1b APPROACHES TO THE DEVELOPMENT OF TRYPANOSOME VACCINE

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The life system of the protozoa is very complicated, though they are unicellular microorganisms, because majority of animals of the group consist of a single cell which is refered to a whole body of an individual. The parasitic protozoa are confined to one or more specific host and compose a distinct life cycle changing their shape and other characteristics implied the term "form".

It is necessary to understand the life cycle of the parasite for correct diagnosis, treatment and prevention of the disease caused by the parasite. On vaccine development, one of an important measure of prophylaxis, we cannot pay too much attention to life cycle of the parasite in selection of the vaccine materials.

Human trypanosomiases are roughly devided into two categories, African (sleeping sickness) and American (Chagas' disease). The former is caused by *Trypanosoma* (*T.*) *brucei gambiense* (Tg) and *T.* (*T.*) *b. rhodesiense* (Tr) which are transmitted by tsetse fly (*Glossina*) and injected into the host with saliva at the bite as is called *Salivaria*. Animal trypanosomiases caused by African trypanosomes and mediated by tsetse, in the name of *Salivaria*, are also quite important in the concequence of reduction in food supply when live stocks and game animals are spoiled. The trypanosome speciese concerned are *T.* (*N.*) *congolense* (Tco), *T.* (*D.*) *vivax* (Tv) and *T.* (*T.*) *b. brucei* (Tb). Tb, Tg and Tr are relatives included in *T.* (*T.*) *brucei* as subspecies.

The latter, Chagas' disease, is caused by *T.* (*S.*) *cruzi* (Tcr) which is transmitted with feces of reduviid bugs (*Triatoma*) as is classified in *Stercolaria*. By the fact stated above and many other differences in life cycle specific to subgenus and species, "trypanosomes" are so much in variety as enable to mention them all together uniformly. In this occasion, African trypanosomes are mainly discussed because they undergo characteristic antigenic variation.

Salivarian trypanosomes appear to have a simple life in mammalian host because they express only one form of trypomastigote form or blood form living in extracellular fluid without invasion to host cells. However, trypanosomes avoid host immune response with

antigenically variable surface glycoprotein (VSG) which is a single costituent of surface coat covering the cell. Trypanosome population expressing new VSG replaces former one, which has been eliminated by host's immune reaction. This replacement occurs cyclically during infection showing waving parasitaemia. The possible number of VSG expressed is estimated to be between 100 and 1,000. It can be concluded that it is not practical to prepare polyvalent vaccines against to such a large number of VSG. Cross reactive region of VSG is detected but is cryptic, hence acquired immunity is only VSG specific. This intricacy made blood form assumed to be unsuitable for effective source material of vaccine and vaccine development to be hopeless. Though, remarkable improvement in cultivation of African trypanosomes from 1976 in ILRAD by Hirumi and his coworkers raised the possibility of devising a vaccine. The life cycles of the parasites were completed *in vitro*. This suggested any stage of the parasites might be produced in a desired amount, as well as metacyclics, in culture vessels. In addition, it has been reported by others confirmably that blood forms of any antigen type of VSG ingested by tsetse lose their surface coat, at the end of cyclic change infective form of metacyclics reacquire the surface coat and at this stage VSG would revert to restricted metacyclic VSG (M-VSG). If this is true, protection agaist infection by the vector appears to be easier. Taking one more reason into account that metacyclic form is the first to be introduced to mammal hosts in much smaller number at infection, metacyclic form turned to be promissing as the source material of vaccine when *in vitro* propagation of metacyclic has become feasible.

Though the life cycle of human infective Tg and Tr has been completed *in vitro*, the progress in studies of these parasites is still behind to animal infective trypanosomes in production of metacyclics in culture. *In vitro* propagation of metacyclics in semi-large scale has been achieved for Tc at first and recently for Tv and Tb by Hirumi and his members. Especially *in vitro* produced metacyclics of Tco were broght to some practical experiments. Calves infected with metacyclics from culture and treated with trypanocidal drugs resisted to challenge with infected tsetse and inoculation of sonicated metacyclics from culture with adjuvant manifested protective immunity in goats.

New findings reported since late seventies proposed the reconsideration on the competence of metacyclics for the source materials of trypanosome vaccine. Some of them demonstrated the heterogeneity of VSG which had been supposed to be single referred to a "basic antigen". For example, at least 8 and 12 M-VSGs were reported for Tr and Tco respectively. The diversity found even in M-VSG is critically unfavorable to that strategy for vaccine. The other findings may make the situation worse because the reports on Tb cumulatively exhibited possibility of sexual process or genetic exchange between trypanosomes during cyclical transmission and vast antigenic variation potential.

To overcome the mechanism evolved by the parasite it is necessary to find some antigenically conservative molecules which play a role of important function on the parasite cell surface and modify them to be immunologically exposed to host.

About Tcr, though antigenic variation is not admitted, trypanosomes change the form from trypomastigote to amastigote and multiply after invasion to host cells. Trypomastigote appear in bloodstream by transformation from amastigote. Infection is initiated by invasion of metacyclic trypomastigotes discharged with feces from the vector. Any trypomastigote dose not undergo cell division. Cultivation of trypanosomes of this species was successful in very early days compaired to African trypanosomes and stage specific antigens have been

detected in some number but effectively protective immunity is not experimentally demonstrated yet. One of the most difficult problem in vaccine development is due to the fact that the disease caused by Tcr progresses by some secondary reaction of the host side rather than direct dammage caused by parasites.

2 VACCINES FOR BACTERIAL DISEASES

2a CHOLERA VACCINE AND TYPHOID VACCINE

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Recently, several effective bacterial vaccines have been developed and field trials have yielded successful results. Those include the cholera vaccine which is a combined vaccine of B subunit of cholera toxin and heat- and formalin-killed cholera vibrios (LPS) and the typhoid vaccine which uses a *gal* E mutant of *Salmonella typhi* (strain Ty 21a). Field trials of Vi polysaccharide vaccine against synthetic peptides derived from the B subunit of cholera toxin have also proved to be protective antigens and hold promise to be used as vaccines.

2b LEPROSY VACCINE, PRESENT AND FUTURE

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Mycobacterium leprae is the etiologic agent of leprosy which afflicts 10 to 15 million people in the world, mainly living in developing countries. Because *M. leprae* has not been successfully cultivated *in vitro*, biologic and immunologic studies of *M. leprae* has been severely restricted.

BCG has been widely used for the prevention of tuberculosis and is reported to be also effective against leprosy to a variety extent depending on the trials. Recent work by Convit and co-workers indicate that the injection of a mixture of live BCG and heat-killed *M. leprae* induce cell-mediated immunity to most lepromatous patients and therefore, is effective as an immunotherapeutic agent. It is also expected that this type of vaccine is more effective than BCG alone for the immunoprophylaxis of leprosy.

Since, the only available source of the organism is infected tissues of the nine-banded armadillo, the supply of the organism will be limited and costly for the large scale vaccine production.

Using recombinant DNA technology, it now became possible to obtain different classes

of protein antigens of *M. leprae* from *Escherichia coli.*

Several studies have revealed that cell-mediated immunity which is regulated by specific T-lymphocyte is required for the protection to leprosy. Although, these protein antigens were found to react with T-cells from leprosy patients or healthy volunteers who received BCG plus heat-killed *M. leprae,* there will be several difficulties to produce large amount of protein antigens from *E. coli* and to induce protective immunity by injecting them. In *E. coli,* it will also be difficult to synthesize complex biological molecule of *M. leprae* which might be required for the induction of protection. The nature of protective antigen has not been elucidated in *M. leprae.*

Stable introduction of *M. leprae* DNA into BCG or other cultivable mycobacteria which have been known to possess antigenically common molecule with *M. leprae* may be a solution of such problem. It is expected that genes of *M. leprae* are expressed much more efficiently in mycobacteria than in *E. coli*, and in addition, host mycobacteria will work as an adjuvant for the induction of cell-mediated immunity. Establishment of hostvector system in mycobacteria thus becomes a major research area in the vaccine development.

Genetic analysis of slowly growing mycobacteria has been extremely difficult since the organism possesses thick cell wall, grows in clumps and no methods for gene transfer have been found.

To develop an host-vector system in mycobacteria, it is necessary 1) to use restriction-less organism as a host bacterium, 2) to develop an efficient means of introducing DNA into mycobacteria, and 3) to construct a vector plasmid or a phage which possess some selectable markers such as drug resistance within the molecule.

Previously, we reported that a rapidly growing mycobacteria *M. smegmatis* ATCC607 does not possess restricition system and can be converted to protoplasts which are required to introduce DNA into the cell by polyethyleneglycol assisted transformation. We also succeeded to regenerate the protoplasts into original rod shaped bacterial cells. BCG, on the other hand, has some restriction enzymes. Therefore, it is necessary to isolate restriction-less mutants from BCG. By mutagenization with nitrosoguanidine, we have succeeded to isolate mutants which do not restrict phage multiplication. Using the mutants, we are now trying to find an efficient method to introduce DNA into BCG.

With regard to the vector-plasmid, both *M. smegmatis* and BCG do not possess any plasmids. Therefore, we looked for the candidate plasmid from other mycobacterial species and found that some strains of *M. fortuitum, M. avium* complex and *M. gordonae* harbored plasmid DNAs. However, we also found it necessary to incorporate drug resistance markers into these plasmids because all of them were cryptic. We are now trying to incorporate drug resistance genes of *E. coli* or *Streptomyces* plasmids into one of such mycobacterial plasmid.

In addition to the research on molecular genetics, future work on the chemical and antigenic analysis of *M. leprae* in relation to protective immunity, on the mechanism of unresponsiveness in lepromatous patients, and on the pathogenesis and virulence of *M. leprae*, will contribute to the control and management of leprosy.

3 VACCINES FOR VIRAL DISEASES

3a DENGUE VACCINE

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Dengue virus infection has been prevalent in many tropical countries, and its spill to temperate areas and infected cases of visitors to tropical countries have been threatening the people in temperate areas also. The public health significance of dengue virus infection has been augmented since outbreak of severe manifestation of dengue haemorrahgic fever (DHF) and increasing number of patients and epidemic areas. Because environmental sanitation requires economical support and vector control is not always reliable and long lasting, dengue vaccine development has been required to provide "a magic bullet" to control dengue virus infection. Soon after dengue viruses were isolated, attempts have been made to obtain attenuated vaccines by serial passages of the viruses in mouse brains. These "classical" dengue vaccines, although they were quite attenuated and conferred immunity to the recipients, could not meet requirements by the Food and Agriculture Department in the US. Attenuation of dengue viruses in cell culture systems without mouse brain passages was conducted in the US and Thailand, and the vaccine viruses were characterized by several *in vitro* and *in vivo* markers of attenuation. Type 2 dengue (D2) vaccine developed in the US was fairly well-attenuated and showed quite good antibody response in yellow fever (YF)-immune recipients, but its lower seroconversion rate and antibody titer in YF-nonimmune recipients along with some side reactions made the vaccinve to be regarded not an ideal one. D2 vaccine developed in Thailand gave quite good response with slight side effects in the recipients both immune or nonimmune to Japanese encephalitis virus, although antibody titer was higher in the former than the latter. In contrast, type 4 (D4) and type 1 dengue vaccines developed by similar methods showed adverse reactions in the recipients, and *in vitro* or *in vivo* markers of attenuation did not parallel to the human virulence. Because second infection with different serotypes of dengue virus has been considered as a "risk" factor to trigger DHF, dengue vaccine should be developed for all 4 serotypes to protect the disease. Even when all 4 types of dengue viruses have been attenuated, they could possibly interfere each other in the recipients.

In order to overcome these problems, development of the second generation dengue vaccine has been planned by the WHO. The strategy consists of the following objectives. (1) Identification of protective epitopes, especially on the virion envelope glycoprotein (E) and the nonstructural protein (NS1). (2) Expression of these epitopes by recombinant DNA technologies in prokaryotic and eukaryotic systems to examine their immunogenicities. (3) Identification of virulent factors in viral genes by comparative nucleotide sequencing of attenuated and their parental strains as well as many strains. (4) Development of genetically engineered attenuated vaccine by constructing infectious complementary DNAs of YF and dengue viruses and their genetical manipulation.

Recently, a study group at the National Institute of Health in the US reported successful expression of D4 virus E and NS1 genes by recombinant vaccinia and baculovirus systems. They showed that the mice immunized with the extracts from mammalian or insect cells infected with the recombinant viruses were protective against lethal intracerebral challenge with D4 virus. These findings are very promising to develop the second generation dengue vaccine. But the gene products should be purified in large amounts for use as "vaccine" in the future. Even when these problems are overcome, the possibility remains that the immunogene(s) might induce "enhancing antibodies" which could trigger DHF in the second infection. This problem will be avoided by giving high levels of protective immunity to all 4 types of dengue viruses by massive amount of immunization, or by identifying some protective epitopes which do not induce enhancing antibodies, or other epitope(s) which cross-protect all 4 types of dengue viruses.

3b EXPERIENCE WITH FIELD TRIALS OF RHESUS ROTAVIRUS VACCINE MMU-18006

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Rotaviruses have been implicated as the major cause of viral gastroenteritis in infants and young children under 2 years of age worldwide. Although deaths are not usually associated with rotavirus gastroenteritis in developed countries, the severity and high mortality of this disease seems to be a serious problem in less developed countries according to numerous studies in various nations. Therefore, the development of an effective vaccine for infants is highly desirable. In this view, the WHO Diarrheal Disease Control Program has been promoting the development of the vaccine.

Several candidate vaccines derived from human and animal rotaviruses have been developed and tested for safety and immunogenicity as well as its efficacy. Rhesus rotavirus strain MMU-18006 is one of those candidates. Several cross-sectional studies of this vaccine have been performed in order to evaluate its efficacy and safety for infants and children.

The placebo-controlled study of the rhesus rotavirus (RRV) vaccine MMU-18006 strain was conducted at an orphanage in Saporro in an attempt to observe its transmissibility and immunogenicity. Twenty-four infants under 15 months of age, living in the same room, were divided into two groups at random. Thirteen infants were given 1 m*l* of 1:100 diluted vaccine (containing approximately 10^4 PFU vaccine virus) while 11 infants received placebo preparations as controls. There was no significant difference between vaccinees and controls in clinical observations postvaccination.

Fecal specimens were collected daily from all infants of study groups for 3 weeks, and were examined for viral shedding by rotary culture method of MA-104 cells. Nine of 12 vaccinees who could be tested shed virus in their feces but none of controls was positive for viral shedding. Therefore no evidence was available to indicate the transmissibility of the vaccine among study infants.

Nine of the 13 vaccinees had a four-fold or greater rise in neutralizing antibody response

against vaccine strain, and acquired the protective level of antibody titer (1 : 128). Seven of those seroresponders to RRV also showed remarkable seroresponses against serotype 3 of human rotavirus (HRV). However, only 2 vaccinees showed concomitant antibody responses against serotype 2 of HRV. Additionally, acquired neutralizing antibodies gradually decreased within a year following vaccination.

Our results indicate that a booster dose of this vaccine would be necessary for prevention of rotavirus gastroenteritis during first 2 years of life. To cover 4 serotypes of HRVs a polyvalent vaccine would be necessary to be developed.

Panel Discussion
Introspective and Prospective View of Cooperative Medical Projects Experienced in Tropical Countries

1 STATUS OF RURAL HEALTH AND MEDICAL CARES: HUMAN LIFE AND NATURAL ENVIRONMENT IN SARAWAK, EAST MALAYSIA

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Borneo is the third largest island in the world and includes three different countries, Malaysia (Sarawak and Saba States), Brunei and Indonesia (Kalimantan). The area of Sarawak is 124,449 km^2 and 7 administractive divisions. According to the 1980 census figures the total population of Sarawak is 1,250,000 and about 80% of them live in rural areas. On the ethnogeographical grounds the indigenous people in the state could be divided into the coastal groups (Malay, Melanau, Kedayan and Belait), the lowland group (Iban, Kenyah, Baketan, Kayan, etc.) and the upland group (Kelabit, Punan and Murut). They usually live in different types of long houses along the river banks. About 90% of the area is covered with tropical rain forests and there are many rivers, and most of them originated along the border with Kalimantan. The rivers with their innumerable tributaries form an extensive network of waterways which are utilised for travel in the rural areas by large express (long boat) and small boats. Due to lack of motorable roads, overland movement is difficult in outlying areas. This is a major cause for the difficulty of providing medical services in the rural areas.

Prior to the second Malaysia plan period (1971-1975) rural health services in the state of Sarawak were provided through a chain of static dispensaries, sub-dispensaries, travelling dispensaries and maternal and child health clinics. The maternal and child health clinics were built and provided by the local councils while the dispensaries and sub-dispensaries were administered by the medical department in Kuching. In the second and third Malaysia plans, a new integrated system of promotive, curative, preventive health education and rehabilitative services were introduced through the establishment of Pusat Kesihatan and Klinik Desa throughout the state. A Klinik Desa serves a population of 1,500 to 2,500 covering an area of 3-7 miles radius and has the following staffing pattern: One hospital assistant, junior hospital assistant and rural health supervisor, and two public health nurses and attendants, one rural health supervisor. One vehicle driver and outboat driver are included. Pusat Kesihatan is next higher unite with 3 Klinik Desa under it. It has a function providing care for a service area of 3,000 to 25,000 population and supervising the 3 Klinik Desas under it. Provision was made to put a medical officer into serve this centre but due to a shortage of medical doctors, it has been temporarily suspended. It consists of the following staff: one senior hospital assistant, dental nurse, health inspector, public health nurse and junior labora-

tory technician. Top priorities in rural health services were given to the expansion of rural services to the unserved areas, static dispensaries, subdispensaries and isolated maternal and child health clinics may be up graded into Pusat Kesihatan. Flying doctor services are also operated for the serious patients in remote locations. Besides the medical care, preventive, promotive, health education and emergency evacuation, the flying doctor services also serves the department in strengthening the supervision of the peripheral staff, the distribution of drugs, vaccines and other medical supplies including facilitating diseases control activities. In 1983, there are 8 Pusat Kesihatans, 71 Klinik Desas, 19 static dispensaries, 55 sub-dispensaries, 87 village health teams and 21 travelling dispensaries in the 7 divisions of the state and they are well functioning as an important rural medical and health service system.

A look at the incidence of communicable diseases in 1983 shows that gonococcal infection is at the top with 2,572 cases. Measles and tuberculosis come next with 2,305 and 1,678 cases respectively. Malaria (803 cases), chicken pox (701), food poisoning (561), typhoid (337), mumps (286), infectious hepatitis (262) and dengue fever (171) completed the list of the top 10 communicable diseases.

I am indebted to Dr. Stalin Hardin, Director and Mr. Chang Moh Seng, State Medical entomologist of Medical and Health Department, Kuching Sarawak, for their warm-hearted understanding for carrying out field research in Sarawak.

2 CENTRAL AND SOUTH AMERICAS-CULTIVATED BETWEEN WILD NATURE AND IBERIAN CIVILIZATION

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While reflecting my own experiences of investigations and technical cooperation projects in the past in Central and South Americas, it became necessary to cast critical eyes on our way of approach and mode of behavior in there to promote and encourage future similar projects. From this view, I feel it primarily important to understand the culture, people and nature of these areas. In this presentation, I would like to introduce roughly the characteristic features of Central and South American societies.

A vast territory between Guatemala, Central America, in the north and Argentine/Chile, South America, in the south is included in this geographic category which is characterized by the diversity of the climatic zones and topography. The residents inhabiting there also show diversity in races and civilizations. Indigenous residents of this continent are mongoloids which are now distributing all over the American continents particularly in the mountainous and silvatic regions. In the Venezuela/Brazil border area, for exapmle, Yanomami or Yanomama tribes are anthropologically well known and are residing there isolated completely from modern civilization.

The most remarkable feature of the society in this continent has been formed and held since the era of the Iberian CONQUISTADORES (conquerers). At the present, social and political uneasiness and economic instability are the common features, despite their rich

natural resources, lands and economic potentials. It may be considered that such problems essentially derived from the traditional social structure and civilization cultivated since 16 century in this continent. Every country consists of a minority of rich caucasoides (Spanish majority) and a majority of poor meztizos (mixed) and mongoloides/negroides. In a recent issue of National Geographic Magazine (June, 1988), you will read "Guatemala, a fragile democracy" which aimed at the analysis of fragile political-democratic-situation seen in this small country standing economically on the conservative FINCA (plantation) system of the agriculture. Oligo- or mono-culture in the agricultural industry is thus the marked feature of this continent except some industrialized countries. Higher prevalence of analphabetism and the presence of INDIGENAS (American indians) are another problems for many countries from the viewpoints of economy, public health, and industry of the nation. Actually, Latin America is a big mixture of wild nature and civilizations of Europe particularly of Iberia and Africa. The characteristic feature of Latin American culture is a marked individualism which derived from its history and social system. The core of the culture consists of catholicism, self assertion and loyalty to reliable family/friend members. Usually solidarity with the organization or community is not so strong. They are essentially epicurean and consider their profession as a tool in the "Corta vida" (short life). The important concerns in the life are honor, profit, comfort, love and so on.

In order to have efficient relationship with Latin Americans and its communities which are certainly different from us, rhetoric in the communication is very important. The regions are the so called low-context society. Linguistically, Japanese has no similar rhetoric in the mode of communication seen in Spanish and historically we passed without similar custom. Thus, in order to communicate efficiently, we should pay special attention on this point. Secondly, we have to pay appropriate respect to the honor of our counterparts frequently and should not criticize carelessly. Because generally they have been grown differently from the discipline of Japanese type. Thirdly, we should pay attention to the importance of sophistication whenever we intended to have intimate communication to each other. A plain "technician" is not a respectable person in Latin American civilization. On the contrary, there are frequently similar sentiment between Latin Americans and Japanese, and both of us frequently give similar evaluation for various matters. For example, "machismo" is rather notorious in there, while its essence is quite similar to "bushi-do" in the definition. Machismo does not simply imply the shortage of respect to woman, but the discipline of man. Thus, they are far sympathetic than Anglo-Saxons for Japanese. Further, generally speaking, Latin Americans are with less racial prejudice. So, standing on the recognition that Japanese people have been rather peculiar in the history and culture in the world, we will be able to maintain favorable relation with Central and South Americas. For the present, Hispanic culture is gradually spreading into all over the North America (United States and Canada). For this reason, from the strategic viewpoint of the state, cooperations in any fields with Latin America will be more important. Any cooperative projects in research or operation in the future may thus require further understanding of this region from sociological and historical views.

3 GEOGRAPHICAL PATHOLOGY AND MEDICAL ANTHROPOLOGY IN AFRICA

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I. Some problems of scientific methods in field work

In the modern medicine molecular biology has developed very rapidly, and has been emphasized as an important science in medical field. However, one must be aware that macroscopic scientific methods such as epidemiology and geopathology have taken a great deal of roles in medical history. Of almost all the morbid hazard, humankind have been able to get a thread of resolution of the problem by macroscopic scientific methods.

According to our basic philosophy, thinking way and results of our research work, evolutions and manifestations of human diseases are quite frequently and strongly affected by external factors. Some of the factors are as follows: 1. geographical distribution and historical migration of the diseases, 2. virulence of parasites or disease agents, 3. transmission routes of disease agents, 4. host constitutional factors on disease evolutions and manifestations, 5. host-parasite relationships, 6. natural environment of the life and environmental physiology, and 7. human ecology (socio-economical factors, life history, manners and customs, food habits, agricultural and pasturage systems and methods, etc.).

We are conducting medical investigation on Kaposi's sarcoma and retrovirus related diseases in Africa. For pathological investigation into the diseases we have to get information about geographical distribution and incidence, ethnical distribution, sex distribution, age distribution, correlation with other diseases, natural environment including climate, and animal and plant ecology, and human ecology. The problem is that one cannot get accurate demography of the areas where one would plan to examine. Occasionally one can get only an estimation of the population. One of the methods we are taking at the moment is counting the incidence of the diseases among the surgical pathology materials at the hospitals. It is very difficult to bet the real population. Therefore, we have to take the methods of epidemiology and even human and animal ecology.

Some of the possible methods to use in field work are as follows:

1) An air photograph of the resident areas
2) Following up the each case
3) Observation of the disease not only we investigate but also the other
4) Making a field inspection on foot by ourselves
5) Operational research
6) Both field work and laboratory work
7) Cooperational work with investigators in other research field

II. Contact with inhabitant poeple

There are two aspects of the scientific methods in the field of cultural anthropology; "emic" and "etic". The former means "the point of view" of the inhabitant people, and the latter means "the point of view" of the researchers. The purpose of investigation research in the field work should be how to translate "emic" into "etic". In 1960s, medical anthropology has been developing in the United States. And that was the new academic attempt which

they investigate medical systems of native people on the side of their "emic". Tropical medicine is historically a part of western medicine itself. However, on the aspects of treatment and therapy, tropical medicine may be their endeavor to combine two medical systems both "emic" and "etic" together. I think medical anthropology should not be only treatment or health care of the inhabitants in the tropics but also "medical science" itself.

One of the another important points is how to contact with the native researchers as counterparts in the tropics. We must have continuously collaboration work with the researchers there. One of the best ways is to make native researchers be principal investigators of the some research groups of our proposed projects or the first author of the papers of the work.

Finally, it is unable to neglect the political problems of the world. It is inevitable to consider about the international problems when you perform some projects internationally. We must always be interested in moral of medical researchers as well as human history and society, and current topics of the world.

4 BACTERIOLOGY PROJECT IN KENYA MEDICAL RESEARCH INSTITUTE—PRESENT STATUS AND PROSPECTIVE VIEW—

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The most outstanding of the facilities built through external assistance in the KEMRI Headquarters and Central Laboratories complex which was constructed through a grant aid from the Japanese Government to the Kenya Government.

In considering the pathogenesis, prevention and treatment of bacterial diarrhea from the ecological standpoint of infectious diseases, conducting an analysis of bacterial species comprising the intestinal flora should be emphasized as a fundamentally critical objective.

Through the implementation of KEMRI/JICA technical cooperation project, it would be emphasized that good correlations between Japanese experts and their KEMRI counterparts should be maintained. Good communication oriented direction between them is one of important factors to obtain the fruitful result in KEMRI/JICA project. The communication is a two way process and there should be mutual encouragement and effort on either side to interact more with each other.

The success of international exchange depends on understanding different culture and history between us from the standpoint of common way of thinking.

5 JICA TECHNICAL COOPERATION PROJECT AT RESEARCH INSTITUTE FOR TROPICAL MEDICINE, PHILIPPINES AND ITS PROSPECT

YOSHINORI KANEKO
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Research Institute for Tropical Medicine, Philippines, is one of the Grant-in-Aid Project of JICA, which was completed in 1981 and followed by Technical Cooperation Project for 7 and half years.

The subjects for Technical Cooperation were acute respiratory infection including immunization, diarrhoea, and others. The development of virus laboratory technology was the most important one. The Institute had additional aids of Animal Experiment Laboratory in 1984 and two buildings for training and residence for trainees and visiting staffs in 1988. The annual budget for technical cooperation was 50 Million Yen for 7 years. At the time of termination of technical cooperation project the Institute had the total of 370 staffs, the annual budget of 20 Million Peso from the Philippine Government, and getting the total of around 70,000 US-dollars equivalent for research from WHO, United States, Canada, Australia and others.

The Institute has Departments of Microbiology, Medical Entomology, Immunology, Epidemiology, Pathology, Biochemistry, and Clinical Research Department including 50 beds, and Animal Experiment Laboratory.

The total of 28 JICA experts including experts for virus (7), operation of laboratory equipments (4), Medicine (3), Pediatrics (3), Experimental animal (3) Bacteriology (2), Medical Entomology (2), Tuberculosis (1), and others (3). Some of them cooperated two or three times. On the other hand the total of 22 Philippine-counterparts were sent to Japan for study.

Many of the senior staffs of the Institute had been trained abroad and able. However, because of the shortage of the national budget, the corroborative study, information for action in other words, was not enough.

The patients at the clinical research department were most important for research. The total of 70% of the patients were pediatric age and almost 100% of the In-patient were infectious nature. Only 6% of the patients were able to pay for medical care.

The total of 548 deaths, 19.5%, among 2,808 In-patients for the period of 1984-1986 was recorded and 71.6% of the death among In-patients occurred within 3 days of hospitalization.

The laboratory technology on bacteria was already developed and it was confirmed the ratio of diarrhoea by plural infections to the total was 17% and very high in rainy season, suggesting the contamination of well water in the community.

In relation to EPI, the effort was concentrated on Polio, Measles, and DPT. It was confirmed that the Schick negative rate by age strongly suggested the implementation of EPI was not enough in depressed area of Metro Manila, suggesting the difficult situation due to urbanization. On polio the sero-positive-conversion rate by immunization was more than 80% and HI antibody positive rate against measles of the infant aged 6-11 months was 36.8% and 88.9% at the age of 4 years.

Some problems on the administrative process of the technical cooperation were almost the same as stated in the abstract of Dr. Ozawa. However, the author is convinced the Institute would serve and develop as an information center for communicable disease control in Philippines.

6 PRESENT STATE AND SOME PROBLEMS ON THE BASIC STUDY OF INFECTIOUS DISEASES BY INTERNATIONAL MEDICAL FOUNDATION OF JAPAN

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The basic study of infectious diseases have been conducted once in a year since 1980. The objectives of the study are to gain an understanding of the present state and the recent trends of infectious diseases in developing countries and to conduct studies and analysis of preventive measures, diagnosis and treatment of the diseases. This would lead to the formulation of the most vital projects in the fields of health and medical care, and would provide basic data and specific recomendation. Diseases to be covered by the study are bacterial, viral, protozoal and helmintic diseases. Following major informations are covered:

1. Documents related to the health statistics.
2. Factors influencing the outbreak of diseases and recent trends.
3. Present organization of the administration for health and medical care (central and local) related to the control of infectious diseases.
4. Legislative regulations for the control of infectious diseases.
5. Control measures for infectious diseases (prevention, diagnosis and treatment, including health and sanitary education).
6. Present state of strategies to improve environmental sanitation.

The team consists of four experts specializing in the fields of microbiology and virology, parasitology, public health and health administration, and clinical medicine. Their tasks are as follows:

1. Microbiology, virology and parasitology; (a) Pathogenic factors (distribution, drug resistance etc.), vector animals and insects. (b) Operational functions, physical set up and arrangements of laboratories (including the standarization of examination methods) (c) National-wide supply and quality control of vaccines. (d) Determinations of a future direction towards improvements in technology and system.
2. Public health (investigation of principal measure for controlling infectious diseases); (a) Preventive measures including food hygiene and management. (b) Information system (information obtained from individuals, groups, and laboratory personnel). (c) Human resources, training, and arrangement for assignment. (d) Vaccinations and their legal provisions. (e) Meteorological factors, geographical factors, water supply, problems on sewage disposal, and nutritional status of inhabitants.
3. Present status concerning diagnosis and treatment of infectious diseases; (a) Lethality. (b) Methods of diagnosis. (c) Methods of treatment. (d) Clinical facilities. (e) Current

state of medical equipment.

4. Administrative system and its functions; (a) Information system and its functions. (b) Functions and location of clinical and laboratory facilities. (c) Human resource and training of health manpower.

The study have been carried out on 8 countries (Thailand 1980, Philippines 1981, Indonesia 1982, Bangladesh 1983, Sri Lanka 1984, Paraguay 1985, Nepal 1986, Ghana 1987, and Pakistan 1988). On the basis of our experiences I will discuss some problems.

7 MEDICAL COOPERATION ACTIVITIES CONDUCTED BY JAPAN INTERNATIONAL COOPERATION AGENCY (JICA)

TAKEFUMI KONDO
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JICA is a government agency operating under the jurisdiction of the Ministry of Foreign Affairs. It was established in 1974 under the provisions of the Japan International Cooperation Agency Law. JICA implements programs for the purpose of contributing to the economic and social progress of the developing world. These programs cover a very wide range, centered around government-based technical cooperation.

Technical cooperation, one of the main activities of JICA, is aimed at helping developing countries to develop human resources capable of playing an important role in their nation building. Technical cooperation by JICA in the field of health, medicine, family planning and population consists of training program, expert dispatch program, equipment supply program. The project-type technical cooperation is an integrated technical cooperation program in which the three abovementioned aspects of technical cooperation are combined. This project-type technical cooperation is the core of the medical cooperations. In this field JICA has 29 project-type technical cooperation programs and 6 new projects will start this year. Of these programs Asian countries' share is the largest (18 projects).

JICA's other activities include a grand aid program to extend financial assistance for the construction of various facilities (usually as an integnal part of technical cooperation project) and Japan overseas cooperation volunteers program (JOCV). These two programs cover medical cooperation. JICA has overseas emergency relief system which includes medical relief. When a disaster takes place in a developing country, this system is expected to be arranged immedeately upon the request from the disaster hit country.

Further expantion and change in quality in these programs are necessary in order to improve health status in developing countries.

8 PROSPECTIVE ROLE OF JAPAN IN INTERNATIONAL HEALTH, A VIEW OF A WHO STAFF

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1. Introduction

World Health Organization (WHO) was founded 40 years ago as the first United Nations special agency for health and has receive high requtation for its remarkable achievement, the most outstanding example is small pox eradication. The following presentation is my personal view on the above subject based on the experience as staff of WHO and visiting more than 10 developing countries a year.

2. Current Trend of International Health Activities

In the past there were many researchers and health staff engaged in tropical medicine and public health problems in Japan such as leprosy, tuberculosis and various parasitic diseases including schistosomiasis, filariasis and helminthiasis. They had contributed for improvement of health status in Japan and for progress of medical science in the world. The reason that many medical professionals worked for tropical medicine and public health was simply those communicable diseases and parasitic diseases were endemic then in Japan.

However as those problems have become less and less important, interests in these problems among medical professionals have also gradually been lost.

On the other hand, magnitude of health problems in developing countries remains high. And because of development of close relation among countries in the world, expressed and felt need of international cooperation including health field has rapidly increased. Consequently Japanese Governmental budget for overseas development aid is increasing every year and many bilateral health projects are undertaken involving substantial number of medical professionals.

3. Appropriate Technology and Effective Programme Implementation

It can be said that when the technology was scientifically sound for example effective vaccine or antimicrobials, and programme was feasible, such public health programme succeeded. Therefore both components of the programme, i.e. appropriate technology and feasibility or operationability should always be checked and studied before launching any public health programme.

Even after starting theoritically feasible programme, many operational problems would rise. In such cases as a clinician makes an effort to improve diagnosis and treatment of a patient, so does public health manager the health programme through giving insight to various aspects of problems and daily activities. To do this, he should regularly monitor indicators of activities and outcome of health services and analyze them.

Thus it is obvious that the long term and multi-aspects follow up of international cooperation programme is very important to permit them to evaluate process and ultimate outcome considering local situations. And such activities require a group of various expertise.

4. Prospective Role of Japan in International Health

There is no doubt about importance of international cooperation in health either from humanistic point of view or as one of the ideal types of contribution to the world as Japan must seek for peace as cause given by Constitution. Therefore, Japan should further strengthen international health activities.

However in this circumstances, how are those experiences in individual projects shared with each other and reviewed so that more efficient and effective projects will be planned and implemented? Furthermore does this situation attract more young professionals to tropical medicine or public health? If not, some actions should be taken to change the climate.

5. Possible Approach to Strengthen International Health Activities

In order to meet the increasing need of international cooperation in quantity and quality in health field, the following three points should be considered; (1) organization of coordination committee at national level, (2) formulation of national policy and priorities for international health, and (3) establishment of the system for human resource development in the field of international health.

In order to build up body of medical professionals in international health, the following system should be instituted; (a) to create scholarship programme for training in tropical medicine/international health in other developed or developing countries, (b) to increase research grant in this field, (c) to set up research collaboration scheme with research institutions in developing countries.

In such a way to secure training opportunities we can encourage young medical professionals to join and stay in this field.

Lastly WHO is collaborating with many bilateral agencies in the world including Japan International Cooperation Agency (JICA). We expect this type of collaboration will become more important and meaningful in the future. Besides as immediate benefit WHO can serve as source of information including country situation, reference of technical discussions and various practical advice based on actual programme implementation. Therefore either in individual cases or official mission, WHO service is ready for utilization.

General presentation

1 FOREIGN PATIENTS WITH TUBERCULOSIS FROM TROPICAL COUNTRIES IN TOKYO NATIONAL CHEST HOSPITAL

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Approximately 290 patients with tuberculosis were admitted yearly to Tokyo National Chest Hospital. Recently, foreign patients with tuberculosis from tropical countries, especially from Asia, have been increasing, and therefore we have discussed their clinical and social problems.

From January 1980 to September 1988, 8 foreign patients with tuberculosis from tropical countries were admitted with a tendency to increase, although the number of inpatients with tuberculosis in Tokyo National Chest Hospital were decreasing gradually. Four patients were from the Philippines, one from Burma, one from Indonesia and one from Algeria, and a Israeli patient traveled in India and Tibet. Duration between entering Japan and hospitalization was less than 1 year in 5 out of 8 patients. The age of these foreign patients raged from 18 to 29, with the average of 23.9, the average age of all the tuberculous inpatients being 49.0. All of 8 patients had subjective symptoms, which were cough, bloody sputum, fever, etc. Sputum smears demonstrated positive acid-fast bacilli in 7 out of 8 patients. All the isolates of *Mycobacterium tuberculosis* revealed no drug resistance. Antituberculous chemotherapy was effective in all the patients. All of them showed to have cavities on chest X-ray films. A female Philippine patient's sibilings and a parent were also infected with *M. tuberculosis*. Most patients had past histories of tuberculosis and/or family histories of tuberculosis. Tuberculosis is a very important infection in their home countries.

Immigrants and students from tropical countries have been increasing rapidly and tuberculosis prevails in these countries. Therefore, foreign tuberculous patients immigrated from tropical countries to Japan should be taken account of prevalence of tuberculosis in Japan. International cooperation between a tropical country and Japan would be important measures to improve in tuberculosis control in both countries.

2 STUDY ON BACTERIAL RESPIRATORY INFECTIONS IN CHAING MAI, THAILAND

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KAZUNORI OISHI AND KEIZO MATSUMOTO
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Respiratory infections are common diseases not only in tropical area but in temperate areas. And causative bacteria show different phases according to environmental changes including climates, antibiotics, socio-economic conditions. In the present work, the causative bacteria for respiratory infections in Chiang Mai area (Thailand) were investigated to compare them with those in Nagasaki (Japan). To recognize the difference of causative bacteria and their antibiotic susceptibilities between these two countries will be useful to manage respiratory infections.

Methods:

Place: Chaing Mai Hospital, Thailand

Sample: Sputa from patients with respiratory infections

Technique: Quantitative culture method by using serial ten fold dilutions of the sputum

Period: From December 8, 1987 to January 7, 1988

Antibiotic Susceptibility: Agar dilution method

Results:

Ninety one specimens were obtained from 90 patients with respiratory symptoms in Chaing Mai University Hospital. Of 90 patients, causative bacteria were demonstrated in 37 patients.

Eight strains of *H. influenzae*, 6 strains of *S. pneumoniae*, 5 strains of *B. catarrhalis* and *Klebsiella* sp., 4 strains of *S. aureus* and *β-streptococcus*, 3 strains of *Pseudomonas* sp. and 1 strain of *S. enteritidis* were isolated more than 10^7 cfu/m*l*. Susceptibility of those clinical isolates was examined. All of *S. pneumoniae* showed good sensitivity for ampicillin. Four out of 5 strains of *B. catarrhalis* produced β-lactamase. Two of 8 strains of *H. influenzae* demonstrated β-lactamase. High resistance to ampicillin was found in two strains which was resistant to causative organisms

1) Main pathogenic bacteria of bronchopulmonary infections in Chaing Mai area are a little different from those in Japan. We found *B. catarrhalis, H. influenzae,* and *S. pneumoniae* to be common pathogens in Chaing Mai area as well as Japan. However *K. pneumoniae* was more frequently isolated in Chaing Mai area.

2) Unexpectedly *S. enteritidis* was isolated as causative agent of lung abscess in a compromised host.

(2) Antibiotic susceptibilities

1) All the isolates of *S. pneumoniae* were sensitive to ampicillin.

2) The rate of β-lactamase producing strains of *B. catarrhalis* isolated in Chaing Mai area is approximately as same as Japan. However they were rather sensitive to β-lactam antibiotics.

3) None of the *H. influenzae* isolates produced β-lactamase. Of the eight strains, two showed resistance to ampicillin while ampicillin resistant *H. influenzae* in Janan was mostly associated with β-lactamase.

3 THE COMPARISON OF DRUG-SUSCEPTIBILITY OF *HAEMOPHILUS INFLUENZAE* AND *STREPTOCOCCUS PNEUMONIAE* BETWEEN THE PHILIPPINES AND JAPAN

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H. influenzae and *S. pneumoniae* are important pathogenic organisms causing respiratory tract infections and septicemia in the Philippines as well as in Japan. Recently, the appearance of drug-resistant strains of *H. influenzae* and *S. pneumoniae*, especially to ampicillin (ABPC), are becoming a therapeutic problem in some of the countries. For this reason, we have performed drug-susceptibility tests for these two species isolated in the Philippines and Japan.

The strains used for MICs measurement were *H. influenzae* 98 strains and *S. pneumoniae* 59 strains provided by Research Institute for Tropical Medicine in the Philippines and *H. influenzae* 356 strains and *S. pneumoniae* 179 strains isolated in Japan. Micro dilution broth method with inoculum size of 2×10^5 CFU/m*l* was used for MICs measurement. The drugs used were ABPC, CEZ, CTM, CZX, OFLX, EM, MINO. β-lactamase production of *H. influenzae* was detected by the nitorocefin solution method.

Results and discussion are as follows;

H. influenzae:

(1) Two of 98 strains (2.0%) in the Philippines and 63 of 356 strains (17.6%) in Japan were β-lactamase positive and those MICs to ABPC were all over 1.56 mcg/m*l*.

(2) Five of 356 strains (1.4%) isolated in Japan were resistant to ABPC (MICs>1.56 mcg/m*l*) with resistant mechanisms other than β-lactamase production but none of *H. influenzae* isolated in the Philippines were resistant to ABPC with this mechanism.

(3) Among other β-lactam antibiotics used in this report, CZX was the most potent and the growth of most *H. influenzae* in both countries were inhibited at <0.025 mcg/m*l* of this drug.

S. pneumoniae:

(1) Among antibiotics used for *S. pneumoniae*, ABPC was the most potent and there were no resistant strains in both countries, those MICs were all below 0.2 mcg/m*l*.

(2) Thirty four (19.0%) and 108 (60.3%) of 179 strains isolated in Japan were resistant to EM and MINO respectively, those MICs were over 1.56 mcg/m*l*, but there were no resistant strains isolated in the Philippines.

We have reported that there were few resistant strains isolated in the Philippines, especially to β-lactam antibiotics in *H. influenzae* and to EM, MINO in *S. pneumoniae*. Recently some of the papers reported the appearance of ABPC resistant strains of *H.*

influenzae without any β-lactamase production. This resistant mechanism was considered, in part, due to the changes of PBPs of this strain. Considering the increase in the use of various kinds of antibiotics in the Philippines as well as in Japan, we should pay careful attention to the appearance and increase of resistant strains in these two organisms.

4 ISOLATION OF LEGIONELLA SPECIES FROM THE ENVIRONMENTAL SAMPLES IN THE PHILIPPINES

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Legionella infections have been recognized as one of the important cause of pneumoniae among immunosuppressed patients, and it was appeared that *Legionella* spp. were widely distributed in environment.

October 1987, we investigated distribution of *Legionella* spp. in environment in Metro Manila, the Philippines, where there had been no reports about isolation of *Legionella* organisms either from environment or clinical specimens.

We tried to isolate *Legionella* spp. from 9 samples of cooling tower water and 1 sample of water supplying tower water from 2 hotels and 4 hospitals.

Samples for isolation were inoculated to intraperitoneum of guinea pigs, and onto selective medium (MWY or WYO agar) after low pH (pH2.2, 0.2 M, HCl-KCl) or heat treatment (50°C, 20 min). Only 2 of 9 samples were positive in low pH treatment, on the other hand 5 of 9 samples and 6 of 10 samples were positive in heat treatment and guinea pig inoculation method, respectively. Out of 6 strains of *Legionella* spp., 4 strains of which belonged *L. pneumophila* serogroup 1, one *L. pneumophila* serogroup 3, and one unidentified.

Legionella organisms can grow even in tap water between 25°C and 42°C, and clearly temperature is one of the important amplification factors for the growth of *Legionella* organisms. In the Philippines, the average temperature for through the year is 27°C which may be optimal temperature for their growth. So it is likely that *Legionella* organisms are more frequently isolated from environmental samples in the Philippines than in Japan. Additionally from the fact that 3 out of 4 hospitals were positive culture of *Legionella* spp., and all of two hotels were also positive, there should be *Legionella* infections. So further research are required to isolate *Legionella* spp. from clinical specimen in the Philippines.

5 *VIBRIO FURNISSII* ISOLATED FROM STOOL SAMPLE OF CHILDREN WITH DIARRHEA IN NEGROS ISLAND AND THREE CASES OF PATIENTS WITH DIARRHEA IN OSAKA CITY

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Bacteriological examination of stool from 34 children with diarrhea living in Neglos Island revealed the following results,

1. Four kinds of enteropathogenic bacteria were isolated from 6 cases (17.7%).
2. The species of bacteria isolated were Enteropathogenic *Escherichia coli*, 2 cases (5.6%), *Vibrio furnissii*, 2 cases (5.6%), Enterotoxigenic *Escherichia coli*, 1 case (2.9%), and *Aeromonas hydrophila*, 1 case (2.9%).

And *V. furnissii* was isolated from three subjects of children with diarrhea (11 and 13 years old female and 9 years old male subject) in Osaka City in June 1987. They were treated by *Lactbacillus bifidus* and Fosfomycine that suspectibity was admitted against the bacteria. It was suggested that the enteritis may be associated with over-eating seafood, especially shrimp, ark-shell, turbo and other kinds of shellfish.

6 CHARACTERIZATION OF *VIBRIO CHOLERAE* O1 ISOLATED IN THAILAND IN 1987

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A total of 100 strains of *Vibrio cholerae* O1 were isolated from diarrheal patients hospitalized in Thailand in 1987 and were characterized biochemically. All the strains of *V. cholerae* O1 tested were serotype Inaba, biotype El Tor and Celebes original type in prophage typing. Hemolytic activity to sheep red blood cells was detected in 92% of isolates in Graig test but all were positive in heart infusion broth containing 1% glycerol by the method of Barua and Mukerjee. Out of 100 strains of *V. cholerae* O1, 97 were positive in chicken red blood cell agglutination test and all strains with HA activity were sensitive to D-mannose except one resistant strain to D-mannose. Ninty-nine strains of *V. cholerae* O1 were sensitive to all drugs tested (CP, TC, SM, ABPC, EM and NA). One strain was resistant to ABPC, showing MIC of more than 100 μg/m*l* and to 10 μg/m*l* of 2, 4-diamino-6, 7-diisopropyl-pteridine phosphate (O/129). The MICs against TC and CP were 3.13 and 12.5 μg/m*l*, respectively. Multiply drug-resistant strain of *V. cholerae* O1 was not isolated.

7 EPIDEMIOLOGICAL INVESTIGATION OF CHOLERA INFECTION IN NAKURU (KENYA) IN 1987 AND CHARACTERIZATION OF THE ISOLATED *VIBRIO CHOLERAE*

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Thirty five diarrhoeal samples were examined for O1 *Vibrio cholerae* at Cholera Laboratory in Nakuru General Hospital and Mogotio Health Centre in 1987. Thirteen cases were hospitalized cholera patients and six cases were healthy carriers of *V. cholerae*.

Vibrio cholerae strains were serotyped by the monoclonal antibodies reversed passive latex agglutination assay. All O1 *Vibrio cholerae* were E1 Tor Inaba type, 123 isolates produced cholera enterotoxin.

All O1 *Vibrio cholerae* isolates were resistant to ampicillin, kanamycin, streptomycin and tetracycline, whereas they were susceptible to chloramphenicol and nalidixic acid. Ampicillin, streptomycin and tetracycline resistant, chloramphenicol and nalidixic acid sensitive *V. cholerae* are common in Kisumu, which suggests that they were from Kisumu district (Ehara, 1983). Five strains of Non O1 *V. cholerae* were isolated from river water sample in Mogotio area.

Monoclonal antibodies reversed passive latex agglutination assay is a simple, rapid and easy technique to identify *V. cholerae* in the field work.

8 THE ANALYSIS OF THE INTESTINAL BACTERIAL FLORA OF THE CAMPYLOBACTER ENTERITIS IN CHILDREN RESIDING IN KENYA AND CHARACTERIZATION OF THE ISOLATED ORGANISMS

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In present study, the intestinal flora of diarrhoeal and recovery samples were compaired in *Campylobacter* diarrhoeal children residing in Kenya. The diarrhoeal samples and their recovery samples were collected respectively. Isolation and the bacterial counts of anaerobic

microorganisms, such as *Bacteroides, Bifidobacterium* and *Lactobacillus*, were lower in diarrhoeal samples than in recovery samples. It is essential to maintain a balanced normal intestinal flora, especially anaerobic flora, to prevent or recover from diarrhoeal diseases.

Campylobacter jejuni and *Campylobacter coli* were isolated from bloody diarrhoeal faeces. Three different serotype strains (TCK2, TCK19, Lior20) of *Campylobacter* were isolated from one patient.

Isolated strains showed Penicillin^R, ABPC^S, SM^S, and TC^S.

Two patients seemed to be affected by the family contacts.

9 MICROBIOLOGICAL STUDY OF DRINKING WATERS IN TROPICAL COUNTRIES—FREQUENCY AND SORT OF BACTERIA IN THE SAMPLES THAT THE RESIDUAL CHLORINE IS POSITIVE

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We have carried out the examination of drinking water in tropical countries for 10 years. This survey reports the number of sample which has both residual chlorine and bacteria, and determined the species of bacteria. Water samples were placed into gas-sterilized containers directly. One of water sample was used for water examination and another water sample was used for bacterial test using URICURT which contained CLED and MacConkey medium. We counted the colonies number of general bacteria and coli-form groupes. This survey was based on the data from 1980 to 1987.

The area finding the sample that has residual chlorine is Middle East and this counts for 20%. We found much less samples in the other areas.

This percentage cannot tell you whether bacteria positive level is high or low in the tropical area. The bacterial species were determined by isolated five samples which was found 1987.

We found *Ps. fluorescens* and *Ps. maltophilia* in two samples and *Ps. maltophilia* in one sample and gram positive bacillus in one sample. *E. cloacae* and *Cit. freundii* were found in tap water sample.

According to survey by Dr. K. Takeuchi, there is a possibility that *Ps. fluorescens, Staphylococcus aureus* and *Serratia marcescens* can survive in drinking water of 0.5 PPM residual chlorine. Therefore this implies of possibility that some bacteria can survive in drinking water which has low levels of residual chlorine.

10 STUDY ON HEAT LOSS ABILITY OF PIKA (WHISTLE RABBIT)

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Pikas (*Ochotona rufescens rufescens*, Whistle rabbits) are old-fashioned rabbits which had survived the Age of Ice, and which are called "Living fossil". They have characteristic short rounded ears and weigh less than 300 g. We have reared and breeded pikas since 1985 and reported their higher metabolic rate, higher body temperature and pyrogenic responses to intra-venous lipopolysaccharide (exogenous pyrogen) and to intra-venous recombinant human interleukine-1 (endogenous pyrogen) previously. In the present study, pika's heat loss ability was examined and discussed from the view point of thermoregulation. During general heating (30°C→33°C→40°C, 60% rh), thermal panting which is one of the main heat dissipation mechanism for rabbits was observed in rabbits, however, no increase in respiratory rate was observed in pikas. In 40°C rabbits did not seem to be exhausted, however, pikas lay down on the cage floor with their limbs stretched and sometimes they jumped to the cage wall as if they were confused in a range. Noteworthily, pikas rapidly increased the rectal temperature at heat exposure (40°C, 60% rh) and then finally died after 60-90 min, of which rectal temperature was 44.0°C and 43.1°C, respectively. Labolatory albino rabbits survived this experiment. Though the radiation from the ear surface is another main heat loss mechanism for rabbits, the ear surface area ratio to body surface area in pikas was small compared to rabbits. It was 7.2% in pikas and 17.0% in rabbits. These results revealed the poor heat loss ability in pikas constituted by smallness of the ear surface area and lack of thermal panting.

11 COMPARISON OF HEAT LOSS RESPONSES INDUCED BY LOCAL HEATING OF PREOPTIC AREA AND ANTERIOR HYPOTHALAMUS AND MEDULLARY RETICULAR FORMATION WITH ARGON LASER ON RABBITS

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It is well known that the capability of heat loss in temperature regulation is enhanced by repetitive heat exposure.

In this study, heat loss responses such as cutaneous vasodilation and thermal panting were induced by local heating of the preoptic area and anterior hypothalamus (PO/AH) and the medullary reticular formation (MRF) in rabbits. PO/AH and MRF were locally heated by an argon laser irradiation guided with a glass fiber 0.4 mm in diameter. Then irradiation powers of argon laser (ILP) were within 10 mW to 25.4 mW, and irradiation time was for 10

min at each power. The minimal ILP enough to induce heat loss responses and then temperature changes in the rectum, brain and ear skin as well as in respiratory and heart rates were quantitatively compared in between PO/AH and MRF heating.

The minimal ILP to induce heat loss responses in PO/AH and MRF were 14.5 mW and 19.0 mW, respectively. And there was statistically significant difference in both values ($p < 0.01$). Then maximal rising temperature and volume risen over 0.5°C in the heated area were 1.1°C and 10.2 mm^3 at 14.5 mW, and 1.5°C and 24.3 mm^3 at 19.0 mW. Heart rate (HR) markedly decreased by local heating of MRF compared with local heating of PO/AH. This decrease in HR may be an influence of medullary local heating on the cardiac nerves and cardiovascular center in the medulla oblongata. On the other hand, there were no significant differences in various indicators of heat loss responses except the minimal ILP and change in HR.

Furthermore, heat loss responses were always induced by local heating of PO/AH at 45 mW in the same rabbit, therefore, the present findings are available for research on the mechanism of heat acclimation in central neurvous system.

12 STUDIES ON HYPOTHALAMIC BLOOD FLOW CHANGES IN THERMAL ACCLIMATION

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Hypothalamic blood flow as well as thermal inputs from several thermosensores plays a role in temperature regulation at a neural level in the hypothalamus. Thermal acclimation causes some alteration in the mechanisms of temperature regulation. Therefore, the present study was designed to clarify the influence of thermal acclimation on hypothalamic blood flow. Cerebral blood flow (CBF) of preoptic area and anterior hypothalamus (PO/AH) during general heating and cooling in the heat-acclimated, cold-acclimated and thermally non-acclimated (control) rabbit was measured by hydrogen clearance method. The change in CBF was closely associated with ambient temperature as well as other thermoregulatory parameters. Not only the less increase in CBF due to heating but also less decrease due to cooling was observed in the heat-acclimated rabbit compared to those of control rabbit, and similar results were obtained in the cold-acclimated rabbit. The reduction of gain in thermoregulatory responses occurred during general heating and cooling in both heat- and cold-acclimated rabbit. The present finding suggests that the reduced change in CBF of PO/AH in the process of thermal acclimation might cause habituation phenomenon of thermoregulatory responses to heat and cold stimulation.

13 CHANGE OF SERUM PROLACTIN DUE TO HEAT LOAD AND EXERCISE IN HUMAN

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Serum prolactin concentration is known to be modulated by various physiological conditions i.e. pregnancy, lactation, stress, exercise, sleep, feeding. Especially, hyperprolactinemia due to exercise attracts much attention, which is considered to be a reason of amenorrhea in athletic women. On the other hand, heat load also increases serum prolactin level. In the present study, changes in serum prolactin during heat load and exercise (under 20°C and 28°C) were examined and discussed. Ten athlete students, 5 male and 5 female, 19-23 years old, were subjects. Significant increse in prolactin, $16.0\pm2.3\rightarrow19.3\pm2.1$ (Mean±SE) ng/ml $p<0.03$, due to 40-45°C, 20 min heat load by sauna box was observed. 50% VO_2max bicycle ergometer exercise under neither 20°C nor 28°C condition increased serum prolactin level. There was considerable discrepancy between the results in former reports. It is considered to be due to lightness in exercise intensity, 50% of VO_2max, in this study compared to those in former reports. There was no statistical correlation between serum prolactin and oral temperature. Adrenocorticotropic hormone (ACTH) did not alter by heat load and exercise, which supported 50% VO_2max exercise was light and stressless. Increase in growth hormone (HGH) level due to exercise was observed under 20°C and 28°C. No significant changes in luteinizing hormone (LH) and follicle stimulating hormone (FSH) were observed in any experiment. And additionally, significant decrease in serum prolactin level, $21.7\pm2.3\rightarrow11.6\pm1.8$ ng/ml $p<0.0001$, was observed during 2.3 km, 160 min swimming at 28.5°C of water temperature, which was considered cold stimulus and exercise. These results indicate that light exercise less than 50% VO_2max may not affect serum prolactin concentration and that thermal factor play a role in prolactin regulation mechanism.

14 PHYSICAL PERFORMANCE AND AMBIENT TEMPERATURE —ANALYSIS OF HEART RATE—

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Physical training enhances thermoregulatory function and suppresses body temperature elevation during exercise. Among athletes, considerable interest is attracted to the problem of the relation between thermal stress and physical performance. High ambient temperature above 20°C has an influence on the record in a race and worsens it. In the present study, the

influence of ambient temperature on changes in heart rate during exercise and recovery phase was examined. Ten athletic students, 5 male and 5 female, 19-23 years old, were subjects. 50% VO_2max, 30 min bicycle ergometer exercise was applied under 60% rh, 28°C and 20°C. Initial values of heart rate were 63.4±2.9 (Mean±SE) beats/min at 28°C and 58.8±2.6 beats/min at 20°C, respectively. The values after 30 min exercise were 139.0±4.4 at 28°C and 132.8±5.0 at 20°C, respectively, in which values there exists no statistical significance. However, the value (101.8±5.5) at 31 min (first 1 min in recovery phase) at 28°C was significantly high compared to that (92.3±6.4) at 20°C ($p<0.05$). The value at 60 min (30 min in recovery phase) was 74.8±4.6 at 28°C and 70.6±3.9 at 20°C, respectively, which was significant difference ($p<0.05$). Significant increase in oral temperature due to exercise, 36.78°C→37.39°C at 28°C ($p<0.01$), 36.65°C→37.19°C at 28°C ($p<0.01$), was observed, however, there was no significant difference in increases of heart rate between under 20°C and 28°C. Summarizing the present results, there was no significant differences in the fluctuation of heart rate during exercise between 28°C and 20°C, however, in recovery phase, heart rate at 28°C was higher than that at 20°C. These findings suggest that ambient temperature may affect physical performance.

15 STUDY ON SEASONAL VARIATION OF THERMAL SWEATING

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It is known that sweat volume and threshold temperature for sweating are altered by thermal acclimation. We have reported that thermally acclimatized tropical inhabitants have longer sweat-onset time and smaller sweat volume compared to Japanese. In Japan seasonal variation of ambient temperature is distinct, therefore, it is suspected that thermal sweating response may alter season by season. In the present study, analysis of thermal sweating was performed in both summer and winter, in order to clarify the seasonal variation of thermal sweating. Six healthy men living in Nagasaki City were the experimental subjects. Local sweating at the chest and abdomen induced by local heating (43°C water bath) on lower legs for 30 min in an environmental control chamber (25°C, 60% rh), which was determined by using of capacitance hygrometer-sweat capture capsule method. Mean ambient temperature of experimental period was 26.1°C in summer (July to September) and 7.9°C in winter (January to March) in Nagasaki. Sweat-onset time at the chest in summer was 10.8±1.2 min (Mean±SE). While that in winter was 16.3±2.0 min, which was significantly long compared to summer ($p<0.005$). Sweat-onset time at the abdomen was 11.9±1.3 min in summer and 16.4±1.9 min in winter ($p<0.03$). Total sweat volume induced by 30 min heat load at the chest was 121.23±20.90 mg/capsule in summer. And that in winter was 36.59±11.46 mg/capsule, which was significantly small compared to summer ($p<0.01$). Total sweat volume at the abdomen was 48.46±10.83 mg/capsule in summer and 19.20±5.88 mg/capsule in winter ($p<0.04$). These results is presumable that distinct seasonal variation may exist not only in

thermal sweating but also in various kinds of physiological system such like metabolic, cardiovascular and endocrinological system which is closely associated with comformation of long term thermal acclimation and thermoregulatory habituation seen in tropical inhabitants.

16 A NATIVE TAIWANESE CASE OF SCHISTOSOMIASIS JAPONICA

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It is generally accepted that *Schistosoma japonica* in Taiwan is zoophilic. The reported cases of Schistosomiasis japonica in Taiwan were all mainlanders who came from Continental China after World War II. In this paper we report a native Taiwanese case complicated with colonic carcinoma.

Case: A 66-year-old male, ex-policeman and bywork farmer. Chief Complaints: Bloody stool, anal bleeding and anal pain. Family History: Noncontributory. Past History: An appendectomy 5 years ago. Present Illness: The patient noted difficulty in the bowel movement with occasional bloody stools in January 1986. Anal bleeding developed soon. Anal pain was noted in April. He came to the Chang Gung Memorial Hospital, Kaohsiung on 7/4/86, and was admitted under suspicion of rectal cancer on 7/21/86. The patient came from South Taiwan. He was said to have never traveled outside Taiwan. Admission Note: The patient had malaise, emaciation and anal pain with bleeding. The abdomen was flat and soft. The liver and the spleen were not enlarged. An ulcerative, ill-defined mass was palpated 4 cm above the anal verge. The chest X-ray film was normal. BP 130/90. Hematology, blood chemistry and liver function test were within normal limits, except CEA 26.4 ng/m*l*.

Rectectomy and jejunal tumor enucleation were made on 7/23/86. The patient was discharged on 8/5/86.

Pathological Findings: Sections showed a moderately differentiated adenocarcinoma of the rectum invading the anus, and deeply into the perirectal soft tissue. The jejunal tumor was a leiomyoma. There are also many calcified *S. japonica* ova in the submucosal region and muscular wall of the rectum.

Follow-up: The patient expired on 8/2/87.

The patient was born in Taiwan and said to have never been abroad. It seems likely that the patient was infected in Taiwan. However, previous epidemiological studies on schistosomiasis in Taiwan indicated that *S. japonica* in Taiwan is zoophilic. Neither *S. japonica* nor *Oncomelania* snail has been reported from the patient's native place, Pingtong. There remains a possibility that he was sent as a soldier of Japanese Army to the Philippines or Continental China during World War II, and infected with Chinese strain or Philippine strain. The rice field which the patient had farmed was sold, and changed to a coconut farm. Any snail except *Ampullarium canaliculatus* has not been found there.

17 TREATMENT OF OPISTHORCHIASIS WITH PRAZIQUANTEL

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One case of opisthorchiasis treated with praziquantel is reported with clinical manifestations, laboratory data and liver ultrasonography findings. The patient is a 27-year-old Thai woman from northeastern Thailand and used to eat raw freshwater fish in her birthplace. She was admitted to the hospital because of continuous right quadrant pain, nausea and epigastralgia. Since abdominal X-ray and liver ultrasonography revealed the stone-like shadow in the gall bladder, cholecystectomy and choledochostomy were performed under the diagnosis of choledocholithiasis. During the operation, over ten of adult *O. viverrini* were found in the bile and ectomized organ. Fecal egg examination detected 350-500 eggs/g of feces of the patient. Laboratory investigation revealed eosinophilia (22%) just before the operation, and transient elevation of serum alkaline phosphatase and ZTT, which was slightly suggestive of the obstruction of the biliary duct. A test for hepatocellular and renal function was whithin normal limits. She was orally administered with 50 mg/kg of praziquantel in 3 doses for 3 consecutive days. Side effects as nausea and abdominal pain were observed, but they were mild and disappeared after treatment. Within 1 week after administration, complete clinical cure was obtained and a stool examination revealed no eggs in feces.

18 EPIDEMIOLOGICAL SURVEY OF LEISHMANIA IN ECUADOR AND CHARACTERIZATION OF ISOLATES

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New World leishmaniasis is widely distributed in Ecuador, where it is a considerable health hazard. According to the articles published in Ecuador to date, there may be three or four clinical forms of the disease: cutaneous cases, ca. 93% of the total; mucocutaneous, ca. 6 or 7% and visceral and diffuse cutaneous ones. The last two forms have not yet been

parasitologically proven in the country. The identification of parasites have been done bosed on clinical manifestations in human patients, together with the epidemiological features, the lesion developments in hamster infections and the growth patterns of parasites *in vitro* cultures. We are studying the transmission of leishmaniasis in this country since 1982. In addition to the two known vector species, *Lutzomyia trapidoi* and *Lu. hartmanni* (Hashiguchi *et al.*, 1985), *Lu. gomezi* was added to the list of Ecuadorian leishmaniasis vectors in this survey. With regard to reservoir hosts, *Tamandua tetradactyla* was newly implicated, in addition to *Potos flavus, Sciurus vulgaris* and *Choloepus hoffmani didactylus* had already been listed as leishmaniasis reservoirs. Six strains of *Leishmania*, isolated from wild mammals and humans, were identified by isoenzyme electrophoresis and by their reactivity patterns to a crosspanel of specific monoclonal antibodies using a radioimmune binding assay. Single isolates from *S. vulgaris, P. flavus* and *T. tetradactyla* were identified as *Leishmania amazonensis.* Three other strains, isolated from cutaneous lesions of humans, were identified as *Leishmania panamensis.*

19 CURRENT STATUS OF LEISHMANIASIS AND CHAGAS' DISEASE IN PARAGUAY

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Leishmaniasis and Chagas' disease are serious medical problems in the Central and South American countries. From 1983 to 1987, we participated in a cooperative medical project in Paraguay and carried out epidemiological studies of these tropical infectious diseases.

(1) Leishmaniasis: This disease is found in the eastern parts of the country, among which three regions, San Pedro, Caaguazu, and Alto Parana, are highly epidemic. These regions accounted for about 90% of 1,093 reported cases (MSPBS, 1985). The major clinical symptom was cutaneous leishmaniasis characterized by ulcer formation on the arms, hands, face, legs, and body trunk. Mucocutaneous leishmaniasis was also observed in which the patients exhibited severe damage of the nasal septum, lips, and larynx. These observations suggest that the Paraguayan pathogen belongs to the *Leishmania braziliensis* complex. The damaged foci frequently came in contact with bacteria and/or fungi causing further advancement of the damage. Treatment by repeated injections of Glucantine requires long duration and large amounts of the drug, yielding high-cost treatment for these patients. In addition, there was limited supply of the drug. The spread of endemic areas of leishmaniasis has become an increasing problem. The reasons for this are considered to be as follows: a) the

rapid, vast deforestation for the development of agricultural fields, b) disappearance of natural habitats for animal hosts, e.g., forest rodents, and c) increase in infection among forest workers, immigrants, and seasonal workers who become available as hosts due to the decrease of resevoir animals.

(2) Chagas' disease: This disease is distributed throughout Paraguay, including the western areas (Chaco). We conducted an epidemiological survey of Chagas' disease in La Colmena (a population of about 5,000) Paraguarí. Out of 884 samples collected, 82 sera were judged as positive in IHA and IFA. The seropositivities varied from a lower rate of 0-4.6% in the community of Fátima, in the center of La Colmena, and in the Japanese colony to a higher rate of 16-21% in the communities of Cerrito, Mbocayaty, and Yajhapety. The average seropositivity (9.3%) in La Colmena was lower than that in other highly endemic areas (23%) (SENEPA, 1984). Sixty four of the 82 seropositive cases were examined electrocardiographically, and 20 cases exhibited some abnormalities. On the basis of the abnormal findings of the ECG {complete right bundle brance block, ventricular premature beats (multiform), QRS left axis deviation} combined with echocardiography (left ventricular dilatation) and other clinical symptoms, 4 cases, including three who were past the age of 40 years old, were diagnosed as having chronic Chagasic heart disease.

20 BASIC STUDIES ON MONGOLIAN GERBILS OF SUSCEPTIBLE HOST TO FILARIAL INFECTION (1)

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The mongolian gerbil is used as laboratory animal in the fields of virology, cerebral neurology, endocrinology and parasitology.

Especially, in the parasitology, the mongolian gerbil is very useful as the susceptible host to filarial infection. But, there is almost no report about biological characters in this animal.

We estimated biological characteristics of the animal such as growth curve and reproduction, in order to analyse the infection kinetics of the filarial infection.

We obtained the coat color mutant male gerbil called white spotting type. The animal was covered brown back with white spot on nose, head, neck and abdomen, so we attempted to make an increase in the scale of the colony, white spotting male gerbil mated with agouti female gerbil as parents. After the litter of this parents as F_1, they were mated with littermate.

In 1978, Waring *et al.* reported that coat color mutant gerbil was semidominant autosomal mutant (gene symbol Sp) and homozygotes (Sp/Sp) were prenatal death.

However, in our laboratory, albino type with white hair and red eyes advented in F_3. So we thought albino mutants were autosomal homozygotes.

The weight of new borns was about 3 g, body weight increased to 3 months age and after

that, they reached plateau.

Number of new borns are 1-9 heads per one parents and sex ratio is about 1 : 1. Condition of suckling had better in the case of the housing female with male after delivery.

In the ratio of organ to body weight, thymus is 2 times and adrenal is 4 times larger than those of mice or rats, and this is remarkable character in the mongolian gerbil.

21 BASIC STUDIES ON MONGOLIAN GERBILS OF SUSCEPTIBLE HOST TO FILARIAL INFECTION (2)

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The mongolian gerbil is representative animal to experimental filarial infection. There are many reports to agouti type gerbil infected with filaria. Estimation of these data, however, are not standardized.

Five conventional animals were housed in one cage at 25°C room temperature and 70% moist. Ten adult animals of each sex and each coat color were used in this study.

Blood samples were obtained from the anesthetized animal with ether from the retro-orbital venous plexus by heparinized capillary pipettes. Total red blood cell (RBC) and white blood cell (WBC) counts were determined with microscope. Packed red-cell volume (PCV, Ht) were examined by means of microcentrifugation. Hemoglobin (Hb) levels were determined spectrophotometrically by the cyanmethemoglobin technique. Dry film blood smears were stained with Wright and Giemsa stain and subjected to a 100 cell differential count. The data were subjected to statistical evaluation using the Student's t test.

RBC counts of the spotted and albino gerbils were significantly ($p<0.05$) lower than the agouti gerbil, indicating the presence of a slight anemia. In this phenomenon, Waring *et al.* were discussed that expression of lowered red blood cell (RBC) counts was not sex limited or sex linked. WBC and 100 cell differential counts were not significant among coat colors and between sexes, but in each animal, WBC seemed to varied with physiological factors. Eosinophil counts are a few in dry film blood smears, and this is thought to be possible marker of the infection model with parasite.

Now, we observe to compare the change of that 3 coat color gerbils infected with *Brugia pahangi*.

22 STUDIES ON *IN VITRO* MAINTENANCE OF ADULT *DIPETALONEMA VITEAE* WITH SPECIAL REFERENCE TO THEIR SURVIVAL AND MICROFILARIAL RELEASE

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Survival and microfilarial release of adult *Dipetalonema viteae* were studied. Adult female worms of various ages were maintained in NI medium (a 1 : 1 mixture of NCTC 135 and Iscove's modified Dulbecco's Medium) or NI-20FBS (NI medium supplemented with 20% fetal bovine serum) with the gas phase of 95% N_2-5% CO_2. The medium and the gas were changed every three days. The peak of microfilarial release of 2,000-18,000 microfilariae per female in NI medium per 24 hr occurred about 10 days after the start of culture. Microfilarial release in NI medium declined and ended in general about one months after the start of culture. The adult females moved actively for about 50 days or more and survived up to 82 days in NI medium. The females in NI-20FBS showed active movement for about two months. Some of the worms survived more than 83 days in NI-20FBS. Addition of fetal bovine serum to the NI medium increased the number of microfilariae released and extended the period of release (This research was carried out with the cooperation and under the supervision of Dr. Paul P. Weinstein, Department of Biology, University of Notre Dame).

23 SCHISTOSOMIASIS JAPONICA AND CARCINOGENESIS—A COMPARISON OF TUMOR INCIDENCE RATES IN THE LIVER AND DIGESTIVE ORGANS OF MICE DURING A MUTAGEN (TRP-P-2) FEEDING—

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Association between schistosomiasis japonica and hepatic cancer has long been discussed. Inaba *et al.* (1977) have shown an epidemiological evidence to list schistosomiasis as a risk factor for hepatic cancer in an endemic area in Japan. There are reports that experimental *S. japonicum* infection increased the susceptibility of liver tissue to carcinogen. We investigated whether cancer incidence on the liver and the intestine is facilitated in long-term feeding of Trp-p-2 which is known as a specific carcinogen for the liver. One hundred nine mice of CDF1 (female, 8 weeks old) were divided into four groups. Group 1 (41 mice) were exposed intraperitoneally to 20 cercariae of *S. japonicum* and provided with carcinogenic diet containing 0.02% Trp-p-2. Group 2 (29 mice) were not infected and kept with the same carcinogenic diet. Group 3 (20 mice) was infected with *S. japonicum*, and group 4 (19 mice)

was not. They were provided with basal diet as references to group 1 and 2, respectively. Untill the 57th week of infection, tumor incidence on the digestive organs in group 1 was 13 in 19 mice died. On the other hand, that in group 2 was 4 in 6. There was significant difference between the incidence of the two groups. Histological examination of hepatic tissue at 57 weeks showed the same tendency. Hepatocellular carcinoma was detected in 9 out of 13 mice died with tumor in the digestive organs in group 1 and 2 in 4 mice died with tumor in group 2. All of survived mice were killed at 58 weeks. Hepatocellular carcinoma had developed in all 11 mice in group 1 and 21 mice of 23 mice in group 2 in the liver. Only two mice in group 2 showed hepatocellular adenoma. We could not find significant difference in both groups. The facilitation on the incidence of liver cancer was not observed finally in group 1. It was considered that Trp-p-2 was too strong as carcinogen to examine a weak risk factor of schistosomiasis or observation period was too long to examine the difference.

24 LECTIN BINDING BY MUSCLE LARVAE OF *TRICHINELLA SPIRALIS*

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The topographycal distribution of lectin binding sites on the muscle larva of *T. spiralis* was investigated by means of fluorescen-tagged lectin and colloidal gold-tagged lectin aiming to gain chemical information of each cellular components. Staining with fluorescen-conjugated Con A was granular and the location resembled that described for glycogen. WGA gave similar results but far less intense staining. Under the electron microscope Con A binding sites were observed on the midgut occupying substance, brush border, cord granules, hemolymph and glycogen aggregates all over the worm body, but not on the cuticle and stichocyte granules; WGA binding sites were on hemolymph and glycogen aggregates but not on the cuticle and stichocyte granules. No effective staining was observed with Lentil lectin. GS lectin stained the inner layer of the cuticle especially a region close to the cuticle surface.

25 ALIMENTARY TRACT ASSOCIATED ANTIGEN IN *TRICHINELLA SPIRALIS*

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By means of immunocytochemical staining we show the occurrence of antigenic substance in the lumen of the esophagus and the midgut, the antigenicity can be recognized by Wistar rats, Fischer rats and humans. The esophagus- and midgut-occupying substances and

brush border in the midgut are responsible structures for that antigenicity. By reacting ultrathin sections of LR White-embedded muscle larvae of *Trichinella* with a panel of sera taken from patients with non-*Trichinella* helminthiasis, including anisakiasis, paragonimiasis, gnathostomiasis, fascioliasis, dirofilariasis and trichuriasis, we demonstrate a fairly good specificity of alimentary tract associted antigens for immunodiagnostic use; they had no, or insignificant, cross-reactivity among the serum pool with the only exception of severe trichuriasis serum.

26 STUDIES ON ANTIGENICITIES OF *TRICHINELLA SPIRALIS* (7) WITH EMPHASIS ON THE CHRONOLOGY OF ANTIBODY RESPONSE

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A spectrum of antibody production by Fischer rats with time after *T. spiralis* infection was investigated by means of immuno-gold staining.

Muscle larvae were fixed with a mixture of paraformaldehyde and glutaraldehyde, dehydrated with alcohol, embedded in LR white resin, ultrathin sectioned and treated with infected sera. Those included sera taken 1, 2, 4, 6, 8, 12, 16, 32 weeks after infection. The specific reaction was visualized by subsequent labeling with biotinated anti-rat IgG and avidin-colloidal gold complex.

Specific IgG antibodies against cuticle, hemolymph, midgut occupying substance, hypodermis, glycogen granules and genital primordium began to be detected from 2 weeks after infection; antibodies against EOS and stichocyte $\alpha 1$, $\alpha 2$ granules from 6 weeks; antibodies against stichocyte β granules from 8 weeks. The time of seroconversion, an essential information to design immunodiagnostic method as well as the specificity of antigen, was thus demonstrated.

27 STUDIES ON ANTIGENICITIES OF *TRICHINELLA SPIRALIS* (8) WITH EMPHASIS ON THE LOCALIZATION OF ANTIGENS RECOGNIZED BY THE PATIENTS

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In situ localization of *Trichinella* antigens recognized by the patient was investigated. Sera were collected from trichinosis patients at the time of the outbreak in Mie Prefecture in 1982, and reacted with ultrathin sections of LR White-embedded muscle larvae. A protein A-gold complex was used as a second layer of the staining; therefere detected specific

antibodies are supposed to be IgG and IgM class. The immunostaining positive structures included the cuticle surface, the inner layer, hypodermis, hemolymph, intestinal gland cell granules, the esophagus occupying substance (EOS), the midgut occupying substance (MOS), stichocyte $\alpha 0$- and $\alpha 1$-granules. Immunonegative structures included the esophagus cuticle, nucleus, nucleolus, mitochondria, muscle fibers and endoplasmic reticulum. While certain worm components, such as stichocyte $\alpha 2$-, β-, γ-granules, cord granules and discrete areas in the genital primordial cells, gave positive and negative results depending on each individual examined. Staining results as to glycogen aggregates was chaos devoiding any specific pattern.

28 A COMPARATIVE STUDIES ON THE DETECTION RATE OF *STRONGYLOIDES STERCOLARIS* BY AGAR PLATE METHOD AND THE TRADITIONAL METHODS

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Although test tube culture method for diagnosis of Strongyloidiasis has been widely used, it has recently been recognized that the method is not so satisfactory as has been believed. A new method to detect Strongyloides larvae was reported by Arakaki *et al.* in 1987 (Japan. J. Trop. Med. Hyg., 16, 11-17). In the new method, nutrient agar medium for bacteriological examination was used.

In the present study, the authors examined 780 Okinawan inhabitants to detect Strongyloides larvae by using this new method and 2 traditional methods (MGL and test tube culture).

The furrows left by crawling larvae on the agar plates were found in 135 cases (17.3%) out of 780, and they are regarded as positive findings of Strongyloides larvae. On the contraly, 24 cases (3.1%) and 34 cases (4.4%) were positive for the larvae in test tube culture method and MGL, respectively.

Fifty-four stool samples, known as positive for Strongyloides, were examined by the above 3 methods. The characteristic furrows appeared in all 54 cases (100%). The detection of larvae by the traditional methods did not reach to 100%. In a single examination of the 54 samples, the furrows were found in about 90%, but the traditional methods revealed about 20 to 40% of positive strongyloides. In the subsequent field work, the furrow positive plates were directly examined by low magnification microscope, and the crawling larvae were found in about 80% of the plates.

In conclusion, the agar plate method was excellent to detect Strongyloides from the stools especially in the cases with small number of larvae.

29 ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF SERUM ANTIBODY IN CATS AND RATS INFECTED WITH *PARAGONIMUS HETEROTREMUS*

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Applicability of *P. westermani* (diploid type, collected in Mie Prefecture, Japan) antigen was examined for the detection of serum antibody in cats and rats experimentally infected with Thailand *P. heterotremus*. Cats served as experimental definitive hosts and rats served as experimental paratenic hosts of Thailand *P. heterotremus*. The antibody titers (in absorbance) of infected animals detected by ELISA with *P. westermani* antigen were as high as those with *P. heterotremus* antigen. These findings indicated that *P. westermani* antigen was available for immunodiagnosis of paragonimiasis heterotremus in both the definitive hosts and the paratenic ones.

The antigens recognized with serum samples of cats and rats infected with *P. heterotremus* were also characterized. In SDS-PAGE with *P. heterotremus* antigen, 3 major bands with molecular weights of about 27,000, 17,000 and 15,500 and a few additional faint bands with molecular weights of about 26,000 were detected. *P. westermani* antigen also migrated into 3 major bands with molecular weights of about 27,000, 17,000 and 15,500. In immunoblotting, serum samples of infected cats and rats reacted strongly with the 27,000-dalton band in both *P. heterotremus* and *P. westermani* antigens. The 27,000-dalton band in both lung fluke antigens was also recognized with a monoclonal antibody (A4-1; Sugiyama *et al.*, 1988) against adult *P. westermani*. These findings indicated that *P. heterotremus* possessed common antigen to *P. westermani* and the molecular weight of the common antigen was 27,000. As the infection with *P. heterotremus* strongly induced antibody against the common 27,000-dalton antigen, it was suggested that *P. westermani* antigen could be substituted for *P. heterotremus* antigen in immunodiagnosis of paragonimiasis heterotremus.

30 ANTI-LEISHMANIA ACTIVITY OF INOSINE ANALOGS

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A promising approach for developing chemotherapeutic agents that possess selective toxicity toward parasites is the one based on certain unique features in structure or function of the parasites that differ from those of the host cells. *L. donovani* is incapable of synthesizing purines *de novo* and is thus dependent on the host for the source of purines. Over two hundred nucleoside analogs were tested in anti-Leishmania assay. The results show that and 3'-deoxyinosine[1] and carbocyclic inosineare[2] potent inhibitor for the growth of promastigotes of *L. donovani* and *L. tropica*. In the present experiments, we show that 3'-fluoroinosine (3'-fluoro-3'-deoxyinosine) inhibits the *in vitro* growth of *L. donovani* promastigotes and amastigotes. In culture, EC_{50} value is 1.0×10^{-6} M for the promastigotes. On the other hand, it is less toxic towards mouse mammary tumor FM3A cells: EC_{50} value is 1.9×10^{-4} M. J774.1 cells, a mouse macrophage cell line, cultured in GIT medium containing lipopolysaccharide. The adherent cells were exposed to promastigotes of *L. donovani* for 1 day, and treated with the drug. Drug activity is then assayed by counting the number of infected host cells/100 host cells. The results indicated that 3'-fluoroinosine is active against amastigotes of *L. donovani in vitro*.

1) Wataya, Y. *et al.* (1984): Biochem. Biophys. Res. Commun. 123, 677
2) Hiraoka, O. *et al.* (1986): Biochem. Biophys. Res. Commun. 134, 1114

31 THERAPEUTIC EFFECT OF INOSINE ANALOGS LOADED IN LIPOSOME IN MICE INFECTED WITH *LEISHMANIA DONOVANI*

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We reported last year that inosine analogs (carbocyclic inosine and 3'-deoxyinosine) had therapeutic effect in mice infected with *Leishmania donovani*. Those drugs and F-2B as a new inosine analog loaded in liposome were examined *in vivo* by the same method.

BALB/c mice infected with *L. donovani* were treated with inosine analogs loaded in liposome for appraising the therapeutic effects. The mice infected with *L. donovani* promastigote 2S-15M strain (1×10^8) were treated with 5 different doses of each drug administered by intravenous injection on alternate days. A group of mice administered with saline or liposome without drug was prepared as negative control and a group with Pentostam as positive control. At the end of the treatments for 2 weeks, impression smear of the liver was prepared to determine the parasite load which was expressed as L.D.U. by counting parasites per 1,000 hepatic cell nuclei.

In the mice infected with *L. donovani*, carbocyclic inosine (10 mg/kg) loaded in liposome showed 89% effect of inhibition as compared with the group of mice administered only liposome, while carbocyclic inosine (100 mg/kg) showed 92% effect of inhibition in the previous experiment. 3'-deoxyinosine (5-10 mg/kg) loaded in liposome showed 61-73%

effect of inhibition, while 3'-deoxyinosine (100 mg/kg) showed 63% effect of inhibition in the previous experiment. F-2B (5 mg/kg) loaded in liposome showed 67% effect of inhibition compared with the group of mice administered saline, while F-2B (50 mg/kg) showed 73% effect of inhibition. The inosine analogs loaded in liposome showed 5-20 times high activity as the free inosine analogs.

32 RELATIONS BETWEEN SHAPE AND NUCLEAR DNA CONTENTS OF GAMETOCYTES IN *PLASMODIUM VIVAX* AND *FALCIPARUM*

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The relationship between the shape and nuclear DNA contents of malarial parasites by microfluorometry on DAPI-stained thin-blood-films was studied with emphasis on gametocytes of *P. vivax* (Pv) and *P. falciparum* (Pf). In brief, thin blood films were prepared from 4 cases with Pv malaria and 1 case with Pf malaria, fixed with methylalcohol and stained with 1 $\mu g/ml$ DAPI solution for 15 min. DNA contents were expressed in arbitrary fluorescence units (FU). The FU of ringforms (R) of Pv (n=77) and Pf (n=19) were 10.12±1.43 and 10.07±2.57, respectively. Macrogametocytes (MG) (n=40) and microgametocytes (mG) (n=30) of Pv were distinguished from the size and nuclear position, and the DNA contents were more than that of R (15.35±4.82 and 29.07±17.15, respesctively). Thus mG of Pv have 1-6 times as much DNA value as ringform which is supposed to contain haploid DNA, probably reflecting a various stage of DNA synthesis up to octoploid. Young gametocytes of Pv (n=11) were recognized by the comparatively round shape and sufficient malarial pigments. However it was difficult to distinguish males from females. Nearly all young gametocytes had the diploid value of DNA (20.86±4.23). Exflagellating gametes of Pv (n=10) showed the haploid value (11.26±3.13). MG (n=10) and mG (n=16) of Pf were equipped with more DNA contents than those of R and distinguishable each other by the degree of nuclear DNA condensation. And there was no difference between the amounts of DNA (16.63±4.18 and 15.30±4.66, respectively) in male and female. Young MG of Pf (n=8) were distinguishable from young mG of Pf (n=11) by the nuclear shape. It was suggested that the both young gametocytes of Pf were diploid DNA value (male: 20.59±8.11, female: 18.95±2.85) as well as those of Pv. Although our results closely fits the hypothesis by Sinden (1982) and the results of experimental study by Janse (1987) on *P. berghei* and *P. falciparum*, futher observation on clinical materials using our method will be recomended.

33 SUSCEPTIBILITY OF *ANOPHELES OMORII* TO *PLASMODIUM YOELII NIGERIENSIS* N67

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Anopheles omorii (TM) was examined for its susceptibility to *Plasmodium yoelii nigeriensis* (N67) under laboratory conditions. *An. omorii* is one of the rarest mosquitoes in Japan and the larvae are found in tree holes in mountains (Tanaka *et al.*, 1979). Recently, TM strain of *An. omorii* has been colonized in the laboratory (Arakawa *et al.*, 1988). In paired feeding experiments, Beech strain of *An. stephensi* from India, as a standard was fed simultaneously on the same mouse whenever TM strain of *An. omorii* was fed. It was found that *An. omorii* (TM) was less susceptible than *An. stephensi* (Beech).

34 *IN VIVO* AND *VITRO* DEVELOPMENTAL CONDITIONS FROM OOKINETE TO SPOROZOITE OF *PLASMODIUM BERGHEI*

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Anopheres stephensi was infected with *Plasmodium berghei* by bite of an infected hamster. Temperature shift of infected mosquitoes from 21°C for 24 hr to 25°C and 28°C increased degeneration rate of oocysts and inhibited the maturation of oocysts. *P. berghei* ookinetes were cultured from hamster blood and purified by discontinuous Percoll gradient (0-36%—45%). Purified ookinetes provided very stable infection to *A. stephensi* through membrane feeders. Mosquitoes fed purified ookinetes in phosphate buffered saline (PBS) and then maintained only with 5% sugar could grow infective sporozoites. Fetal bovine serum (FBS), mammalian cell medium, Ham's F-12, and hamster red blood cells supplemented with PBS improved infectivity of ookinetes to mosquitoes and decreased degenerative changes during oocyst development.

Purified ookinetes were resuspended in the culture medium, F-12 supplemented with 10% FBS and subsequently incubated at 21°C for 2-3 days in a candle jar. Microscopic examination showed a few ookinetes with swollen posterior regions like a bacterial spore in better culture conditions. They were considered intermediate forms in the development from ookinete to oocyst.

35 A LONGITUDINAL SEROEPIDEMIOLOGICAL MALARIA SURVEY IN AN AMAZONOUS HYPOENDEMIC COLONY II CASE PLOTTING METHOD ALMING A COMPUTER GRAPHIC SYSTEM

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In malaria control operation, parasite rate has been used to monitor prevalence of the disease in a target community. When parasite rate in an area becomes lowerer after successful control operation, new measure to monitor transmission of malaria is required. It is widely admitted that indirect immunofluorescent antibody (IFA) test is the most reliable method to catch present and past malaria infection. Thus, the method allows monitoring "period prevalence" and is sensitive enough to catch latent foci in hypoendemic community. Tomé-Açu was opened in early 1960's, another epidemics occurred. A successful control has prevented the epidemics since the time. However, increasing malaria cases are now warned by local malaria control authority (SUCAM). Present study was undertaken on the continuous line of malaria sero-epidemiological study worked at Daini Tomé-Açu in 1976. Studies done in 1986 and in 1987 showed a higher IFA positivity rates than that shown in 1976 in the same community. In 1988, epidemiological dynamics of latent malaria prevalence was studied by house to house visit. Personal history was recorded for each individuals together with collection of blood specimens subjected to microscopic diagnosis and IFA test. Totaling 312 specimens were collected covering all age groups of settlers as well as immigrant laborours and their family in the colony. Two distinctive latent malaria foci was found in Daini Tomé-Açu by dotting positive cases on the map. Western area showed *P. vivax* prevalence, and in the central area of Daini Tomé-Açu *P. falciparum* prevalence was demonstrated although microscopic study on blood smear worked nothing for it. Time course dotting of sero-positive cases on the map using a computer will be useful to devise any new local ideas for control unique in the place. Present study will provide baseline data for such kind of practical application of longitudinal examination in the future.

36 IMMNUOREACTIVE PEPTIDSES CLEAVED BY CYANOGEN BROMIDE FROM JAPANESE ENCEPHALITIS VIRUS ENVELOPE GLYCOPROTEIN E

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Purified Japanese encephalitis virus was disrupted by Triton X-100 and centrifuged through sucrose gradient. Envelope glycoprotein E was precipitated by trichloroacetic acid

and dissolved in formic acid and cleaved with cyanogen bromide (CNBr), followed by SDS-PAGE. Multiple bands of molecular weight ranging from uncleaved 54 K to cleaved 8 K were revealed by Coomassie blue staining. Bands corresponding to 39 K, 22 K and 8 K, as well as uncleaved 54 K, were cut out and immunized to mice by 6 weekly intraperitoneal injection with Freund's adjuvant. Anti-JE antibody titer of individual mouse serum was tested by ELISA and neutralization (N) test. Geometrical mean titer of ELISA was highest for anti-54 K, with gradual decrease for anti-39 K, anti-22 K, and anti-8 K, in this order. N-test at 1 : 10 serum dilution was positive in 1/2 anti-54 K, 0/4 anti-39 K, 5/9 anti-22 K and 4/4 anti-8 K sera. Reactivity of these antiserum to each fragment was tested by SDS-PAGE and Western blotting in order to show their interrelationship. Anti-54 K as well as anti-22 K sera reacted with 54 K, 39 K, 22 K and 8 K bands, while anti-39 K sera reacted with 54 K, 39 K and 22 K, but not with 8 K bands. On the other hand, anti-8 K sera reacted only with 22 K and 8 K bands. The results indicate that 8 K fragment, which may carry some neutralizing epitopes, is a part of 22 K but not included in 39 K fragment, while, 22 K and 39 K fragments share overlapping part. From this reasoning and position of methionine residues on the E protein deduced from its nucleotide sequence (Sumiyoshi *et al.*, 1986), a hypothetical position of these fragments on the E protein was postulated.

The 8 K fragment was purified from CNBr-cleaved E protein by reverse-phase high-pressure liquid chromatography and its N-terminal amino acid sequence was determined. Eleven residues showed complete agreement with amino acid sequence deduced from the nucleotide sequence of postulated position of the 8 K fragment.

37 ENHANCEMENT OF DENGUE VIRUS MULTIPLICATION BY THE INHIBITORS OF mRNA SYNTHESIS

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Recently the genome structure of dengue Virus (DV) has been clarified. However, the pathogenesis of DV infection and the mechanism of viral replication have remained to be elucidated. Here we will report an enhancing effect of actinomycin D (Act-D) upon the reproduction of DV.

Dengue Virus type 1 (D1V, Mochizuki strain) was used for the tests. When D1V-infected cells were treated with 1 μg/m*l* Act-D, D-1 viral reproduction was significantly enhanced. HA titers of D1V released from infected C6/36 and Vero cell cultures increased to twice to 4 times of those from control untreated cells. The amounts of viral genomic RNA extracted from Act-D treated materials were significantly larger than those from untreated one. Data of ^{35}S-labeled experiments also indicated that more amounts of D1V-structural proteins were obtained in the Act-D treated cultures. Ten μg/m*l* of alpha-amanitin, an inhibitor of RNA polymerase 11 in eukaryotic cells, showed a similar enhancing effect upon D1 Virus reproduction as Act-D. It appeared that the virus yields became higher when the synthesis of cellular mRNA was inhibited by substances such as Act-D or α-amanitin.

These data suggest a possibility that certain factor(s) produced in host cells retard the viral production processes, and that the synthesis of such factor(s) is inhibited in the presence of Act-D or α-amanitin. There must be several mechanisms for the enhancement of DV multiplication that may lead to the severity of clinical manifestation of dengue, and the phenomena we here describe is probably one of the cases.

38 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) IN THE DETECTION OF HEPATITIS-B SURFACE ANTIGEN IN BLOOD DRIED ON FILTER PAPER

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In an attempt to find a convienient method for the seroepidemiological study of HBsAg in developing countries, Enzyme-Linked Immunosorbent Assay (ELISA) using blood samples dried on filter paper was evaluated. The results of this method were in complete agreement with both those obtained on original sera by RIa and with those obtained on sera by ELISA. We did not experience any false positive or false negative results. In this study the sample dried on filter paper was eluted by 1 m*l* of PBS (pH 7.2). Dilution rates were caliculated by the calibration line of each serum. Dilution rates were between 0.041 and 0.082. The coefficient of variation of absorbance value of 10 samples of each case were between 5.0% and 14.8%, which indicated an acceptable intratest reproducibility of this method. In addition, storage at 20°C, 20°C with desiccants and 3°C for up to 21 days did not alter the sensitivity and specificity of this method. This method will be useful for the seroepidemiological study of Hepatitis-B virus in developing countries.

39 STUDIES ON HEPATITIS WHICH OCCURRED AMONG LONG-TERM SOJOURNERS IN THE DEVELOPING COUNTRIES, AND PREVENTION AGAINST IT

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Investigations were made on acute hepatitides which were contracted during stay in developing countries. The subject of this investigation was members of the Japan Overseas Cooperation Volunteers who stay in developing countries for two years. The age of the subject was between 20 and 35, and mid year population in 1987 was 1687.

For prevention against hepatitis, inoculation of human immune serum globulin (ISG) and

hepatitis B (HB) vaccine (medical staffs only) was started in 1980 and 1982, respectively. Before inoculation of ISG was started, hepatitis A (HA) was showing the highest frequency among hepatitides, however the incidence decreased remarkably after the inoculation of ISG. In 1986 the incidence of HA was less than 0.1%. No cases of HA was confirmed among those who were inoculated with ISG although there were many among who were not. The possession rate of HA antibody before departure was 12.0% in 1982 and 3.5% in 1986.

Twenty-nine cases of HB have been confirmed during the past 7 years. 27 cases were regarded as those contracted during stay in developing countries, while 2 cases were onset from HBe antigen carrier. The rate of sero-conversion of HB marker was 4.5% in 1986. 194 out of 221 who were inoculated with HB vaccine became HBs antibody positive, and no cases of HB occurred among them. Statistical significance in occurrence of HB was recognized between male and female.

Eight cases of non-A non-B hepatitis was confirmed during the past 7 years. Most cases were regarded as infected orally.

When Japanese, especially young people, stay in developing countries, the risk of contraction of acute viral hepatitis is high. For prevention against it, ISG and HB vaccine is important as well as precaution against foods, water, blood and sexual intercourse. In the present investigation, the prophylactic efficacy of ISG and HB vaccine to long-term sojourners in developing countries was proved.

40 PURIFICATION OF AN ANTIHEMORRHAGIC FACTOR FROM THE SERUM OF *DINODON SEMICARINATUS*

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Purification of an antihemorrhagic factor from the serum of *Dinodon semicarinatus*. An antihemorrhagic factor was purified from the serum of *Dinodon semicarinatus* by a series of high performance liquid chromatographies with a TSK gel DEAE-5PW column. The purified antihemorrhagic factor showed a single band on polyacrylamide gel disc electrophoresis. The factor inhibited the hemorrhagic activity of HR1 and HR2, the hemorrhagic factors of *Trimeresurus flavoviridis* Okinawa. The purified antihemorrhagic factor was stable from 0 to 60°C and at the pH values between 2.0 and 11.0. The molecular weight of the factor was estimated to be 59,000 and 52,000 by a gel filtration and SDS-disc electrophoresis, respectively, suggesting that the antihemorrhagic factor consists of a single subunit. No precipitin lines were found for the purified antihemorrhagic factor with the venom of *T. flavoviridis* Okinawa and its hemorrhagic factors, HR1 and HR2.

41 ANTITOXIC EFFECT OF CEPHALANTHIN AND ANTIVENOM AGAINST THE MAMUSHI (*AGKISTRODON BLOMHOFFII*) VENOM

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Varying dilution of mamushi venom (0.1 m*l*) was mixed with 0.2 m*l* (1 mg) of Cephalanthin or 0.1 m*l* of anti-mamushi antivenom, incubated at 37°C for 1 hr and injected intramuscularly into the thigh of mice. The results indicated that 0.1 m*l* of the antivenom neutralized 600 μg (2 mlds) of lethal activity and 75 μg (129 mlds) of hemorrhagic activity of the venom, whereas any significant antitoxic effect of Cephalanthin were observed as compared with the control of venom alone.

The same results were obtained when the venom was previously injected intramuscularly into the thigh of mice before the administration of Cephalanthin or antivenom.

From those results, it is elucidated that Cephalanthin is not effective to neutralize the venom *in vitro* and *in vivo*. Thus, the idea to substitute Cepharanthin for antivenom on medical treatment of envenomation due to mamushi is unacceptable.

42 A CASE OF SEVERE MALARIA TREATED WITH QINGHAOSU

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The herb *Artemisia annua* has been used for many centuries in Chinese traditional medicine as a treatment of malaria. The substance responsible for its medicinal action is called Qinghaosu (artemisinin). Artemether is one of the derivatives of qinghaosu and we attempted chemotherapy of a case of severe falciparum malaria.

A Japanese 28 years old male complained of a high fever during the round trip in Africa one week before coming back to Tokyo. On the day of admission into Tokyo Metropolitan Ebara General Hospital, he developed fever at 41°C, and asexual forms of *P. falciparum* were observed in the peripheral blood of the patient at a density of 17% of erythrocytes. His consciousness was turbid; cerebral malaria was suspected. Liver was palpable by two finger breadth but spleen was not palpable. Ultrasonography confirmed hepatosplenomegaly. Slight jaundice was observed. On the laboratory findings, anemia (RBC 270×10^4, Hb 7.9 g/d*l*, Ht 23%), leukocytosis (15,300/mm^3) and the decrease of the number of platelets (32,000/mm^3) were observed. A considerable degree of dysfunction of liver and kidney was also noted.

Two hundred mg of artemether on first dose was administered intramusculary followed by 100 mg per dose at intervals of 12 hours to the amount totaling 1,000 mg. The asexual stage parasites from blood films were eliminated in 66 hr after starting treatment. Therefore,

artemether amounted to 600 mg, which is the standard total dosage, saved the patient from severity. When the clinical signs were away, the patient was supplemented by chloroquine (1,500 mg), because recrudescence rate of artemether within one month is recorded higher than 10%. No resistance of the isolate to chloroquine was confirmed beforehand by *in vitro* chloroquine susceptibility test.

Quinine has been very effective in treating severe malaria. However we considered artemether as the antimalarial of higher priority than quinine especially in the treatment of a patients with dysfunctions of liver, kidney and CNS, because side effects by quinine occasionally involve those organs. In addition, the half-life of quinine in the blood is 2.5 times as long as that of artemether. Because of the short half-life and rapid action of the derivative, artemether is documented to be remarkably well tolerated in man and also appears to be safe in the cases complicated by heart, liver, and renal disorders, effectiveness against both chloroquine-sensitive and resistant strains of *Plasmodium falciparum* is another strong quality of artemether.

Most Japanese are non-immuners to malaria, hence tend to develop severe malaria if treatments delay. Artemether seems to be a promising new antimalarial with high potentiality to cure such patients.

43 IMPORTED MALARIA CASES RECENTLY DEALT WITH BY US

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There were six malaria cases (4 falciparum and 2 vivax malaria) diagnosed by the Department of Protozoology, Research Institute for Microbial Diseases, Osaka University from January to July, 1988. A patient (41-year-old male) suffered severe falciparum malaria with a maximum parasitemia of $35.4 \times 10^4/mm^3$. All forms including developing trophozoite, young schizonts and gametocytes were observed in the peripheral blood. He and three other engineers visited Yercaud, a local town in India on March 18, 1988 and stayed there for a week to teach how to manage machineries used in the processing of stone. They did not have any knowledge about malaria. Three slept with open windows but without mosquito nets. Two of the three including our case suffured from severe falciparum malaria. Small to medium Japanese enterprises trading abroad are increasing these years and are sending their employers to malaria endemic areas without informing them about malaria and other tropical diseases.

A relapse case of vivax malaria occurred with a Japanese worker at a forestry company in Papua New Guinea. The forestry company provides antimalarial drugs but workers and their families do not want to take adequate prophylactic chemotherapy and rather prefer malaria infections. Therefore, almost everybody including children suffured from malaria during their 5 years stay there. It seems that there are a lot of Japanese malaria patients abroad, most of which are not investigated in statistic studies on malaria incidence in Japan.

It is necessary that all the enterprises trading abroad organize an effective antimalarial campaign to decrease of imported malaria.

44 ONE CASE OF VIVAX MALARIA SUSPECTED TO BE INFECTED IN INDIA

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One case of vivax malaria, which was treated with sulfamethoxazole and trimethoprim, is presented. In December 1987, a 24-year-old Japanese male came back from India where he had been on voluntary service. He had got high fever and treated with chloroquine in August 1987 and there had been no such symptom later in India. In the evening of April 5, 7, 9 and 11, 1988, shivering and headache associated with high fever developed periodically. On April 12, he admitted to Nagasaki University Hospital. Blood smears on admission revealed the presence of *Plasmodium vivax*. The patient was successfully treated with sulfamethoxazole and trimethoprim and primaquine, and there was no sideeffect. Plasma concentrations of sulfamethoxazole and trimethoprim were determined, which reached maximum at 45, 3.5 mcg/m*l* respectively 4 hr after prescription of the drugs and then decreased gradually. From 1972 to 1988, we have experienced 6 cases of maraliasis, which were successfully treated with sulfamethoxazole and trimethoprim. We are encouraged to use sulfamethoxazole and trimethoprim to maraliasis in Japan, where chloroquine and fansidal are not easy to get.

45 THE EFFECT OF HAEMOGLOBIN IN SERUM AS A TRIGGER OF THE TRANSFORMATION FROM BLOODSTREAM FORMS TO PROCYCLIC FORMS OF *TRYPANOSOMA BRUCEI IN VITRO*

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A further study of the factors which trigger the transformation from bloodstream forms (BSFs) to procyclic forms (PCFs) of a pleomorphic clone (GUTat 3.1) of *T. b. brucei* was carried out using an *in vitro* system in which BSFs continued to grow without transforming to PCFs at 27°C as reported earlier.

A total of ten sera obtained from various suppliers were examined and resulted in their

classification into four groups (G-I, II, III and IV). G-I: Four sera supported the continuous growth of BSFs without transforming to PCFs. G-II: One serum induced a small number of PCFs among many BSFs after two weeks of cultivation, but they disappeared in the following week resulting in a BSF population in the final culture. G-III: Three sera supported the continuous growth of BSFs, however, also induced a number of PCFs by day 10 resulting in a mixed population in the final culture. G-IV: Two sera transformed all BSFs to PCFs within a week and the final culture contained only rapidly proliferating PCFs.

One of the sera in G-III was fractionated into 10 equal parts by freezing, thawing and separating by means of gentle pipetting and their effects were examined in the same manner. Fractions of the 1st to the 5th layers were equivalent to the sera in G-I. The 6th layer was equivalent to the serum in G-II and the 7th to the 10th layers to the sera in G-IV. Furthermore, the 1st fractions of another serum in G-III and two sera in G-IV were also found to be equivalent to the sera in G-I while the 9th and the 10th fractions of the serum in G-II transformed all BSFs to PCFs (equivalent to G-IV).

An addition of various amounts of bovine haemoglobin (BHb) in three groups, (1) 0.05-0.2, (2) 0.4 and (3) 0.6-40 mg %, to the culture medium supplemented with a serum in G-I resulted in the same transformation as induced by the sera in G-II, III and IV, respectively.

An addition of 3 or 6 mM of cis-aconitate (CA) in the same manner did not transform the BSF to the PCF.

These results suggest that (1) BHb or its degradation products, most probably trigger the transformation, (2) CA may not play a direct role in the transformation, (3) most commercial sera may contain the factor varying in amount from lot to lot, (4) the factor can be easily removed (or at least minimized) by the natural gravity separation method and (5) sera containing low amounts of the factor may be suitable for the cultivation of BSFs while those containing high amounts may be used for the propagation of PCFs.

46 ULTRASTRUCTURE OF TRANSITIONAL FORMS OF *TRYPANOSOMA CRUZI* BY SCANNING ELECTRON MICROSCOPY

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The ultrastructure of transitional forms between amastigotes and trypomastigotes of *Trypanosoma cruzi* was studied by scanning electron microscope. Two samples were obtained. Sample 1 was the extracellular trypanosomes collected from LIT medium culture after 48 hr of inoculation which was initiated from trypomastigotes from fibrobrast cell (L-cell) culture and passed through C.M. cellulose column. Sample 2 was the intracellular trypanosomes collected from destructive fibroblast cells which were mechanically disrupted by homogenization after 72 hr of incubation. These two samples were fixed in 2% glutaraldehyde for 2 hr for scanning electron microscopy.

The number of each forms of *T. cruzi* in both sample was different as in sample 1, the

percentage of amastigotes, trypomastigotes and transitional forms was about 10, 30 and 60 while in sample 2 is 80, 10 and 10 respectively. Dividing amastigotes were found also in both sample around 0.1% and 0.7% in sample 1, and 2 respectively and were counted in the amastigotes. The free and attached flagella were clearly seen in these amastigotes.

Therefore, here we propose the new concept of the amastigote that it is one form of *T. cruzi* with rather oval in shape, convex at one side and concave at the another, 2.25×1.50 μm in average size and possesses the flagellum both attached and free parts. The attached flagellum originates near the posterior end, runs straight along the convex curve toward the anterior end then protrudes out freely and shortly to become the free flagellum.

Because it is quite impossible to observe the attached flagellum by light microscope or even the short, free flagellum is also very difficult to be seen with improper staining. That is why the amastigote is described with no flagellum.

The amastigote gradually developed to trypomastigote through the transitional forms by straightening combined with the helical movement of the body together with the change in flagellar structure. The transitional forms could be concluded into c-Form, s-Form and sc-Form as following.

c-Form: The body was oval about 2.73×1.51 μm the posterior end was broadly conical with "c" shape of the attached flagellum and short free flagellum. Some showed coiling movement at the anterior end.

s-Form: The body was slightly elongate about 2.85×1.47 μm. The helical twist appeared, the attached flagellum became "s" shape with free flagellum as short as the c-Form.

sc-Form: The body was slightly elongate about 3.14×1.30 μm, the posterior end was taperingly conical with "sc" shape of the attached flagellum and longer free flagellum.

By twisting or coiling with straightening of the body, it formed the trypomstigotes in which the shape and size varied depending on how they moved. Usually the slender trypomastigote was about 15.25×1.42 μm. Whether the shape or size they would appear, the structure of the flagella showed more advanced in shape and length than those of the transitional form.

To become the amastigote, the trypomatigote might round up the entire body together with coiling and hiddening of the flagellum.

47 INFLUENCE OF MONOSACCHARIDES ON THE GROWTH OF *TRYPANOSOMA BRUCEI GAMBIENSE IN VITRO*

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Trypanosoma brucei gambiense adhere on the surface of feeder layer cells and form clusters between these cells. The failure of adherence will always lead to elimination of the parasites. To investigate this phenomenon, the effect of nine monosaccharides among which constitute cell surface carbohydrates, mannose derivatives, lectin, mannan, anti-mouse brain cell rabbit serum and other reagents on the growth of *Trypanosoma brucei gambiense* over

new born mouse brain cells and feeder layer free system was assayed.

Different concentrations of each of the monosaccharides {α-D-(+)-glucose, D-(+)-galactose, D-(+)-mannose, α-D-(+)-fucose, D-(−)-ribose, D-(+)-xylose, D-(−)-arabinose, N-Acetyl-D-glucosamine and N-acetyl-D-galactosamine} and various dilutions of antiserum were added into culture medium which consists of Eagle's Minimum Essential Medium (MEM) with Earle's salt supplemented with 5% fetal bovine serum and 5% new born bovine serum and used in feeder layer system while, a mixture of equal volumes of MEM and Leibovitz's L-15 medium supplemented with bathocuproine sulphonate and 10% fetal bovine serum was used in feeder layer free system.

In feeder layer system 24 hr prior to parasite inoculation tissue culture dishes were seeded with new born mouse brain cells. Parasites used were *Trypanosoma brucei gambiense* Wellcome strain (Tbg W), obtained from blood of infected ICR mouse and separated from blood components by chromatography method. Procyclic forms *Trypanosoma brucei gambiense* TH-1/78E were obtained from cultures. Other trypanosomes used for testifying the experiments were *Trypanosoma brucei gambiense* ILRAD 1582 and *Trypanosoma brucei rhodesiense* ILRAD 1501.

Initiation of the cultures was carried out by either replacing half of the old medium by fresh one or by centrifuging the supernatant and return the pelleted trypanosomes. The parasite density was determined from the supernatant fluid.

Among the monosaccharides tested only mannose inhibited the growth of bloodstream forms of trypanosomes but had no effect on the growth of procyclics. Mannan, lectin and anti-mouse brain cell rabbit serum had no effect on the growth of the parasites. This inhibitory effect of mannose was presumed to have affected the parasites rather than FLC. Thus, competitive inhibition test of glucose and mannose was performed, but even at the ratio of 1 : 40 mannose/glucose could not eliminate the inhibitory effect of mannose. The effect of mannose and 2-deoxy-D-glucose was also detected in feeder layer free system. Furthermore, derivatives of mannose were tested but most of them did not have any effect on the growth of the parasites. However, D-mannosamine hydrochloride and α-D-mannose pentaacetate inhibited the growth of the trypanosomes, but it was speculated that their mode of actions were different from those of mannose and 2-deoxy-D-glucose because they inhibited the growth of both bloodstream and insect forms and regardless of that, toxic effect was detected on the FLC.

Electron micrographs of trypomastigotes cultivated in culture medium mixed with 5 mM mannose or 2-deoxy-D-glucose showed degeneration of the glycosome, nucleus and kinetoplast.

Taking into account, the same mode of trypanosome growth inhibition *in vitro* and the same morphological structure changes of trypomastigotes after treatment with mannose and 2-deoxy-D-glucose, it is assumed that inhibitory activity of mannose on bloodstream forms resembles that of 2-deoxy-D-glucose which inhibits metabolism of trypanosomes partly by affecting some enzymes in Embden-Meyerhof-Parnas pathway.

48 STUDIES OF TWO ENZYME IMMUNOASSAYS FOR EPIMASTIGOTE AND TRYPOMASTIGOTE FORMS OF *TRYPANOSOMA CRUZI*

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This study was undertaken to develop new tools to study *Trypanosoma cruzi*, the causative agents of Chagas' disease. Every three forms in the life cycle of the Tulahuen strain of *T. cruzi*, epimastigotes (EPI) the living form in the vector Triatomine reduviid bugs, trypomastigotes (TRP) the intertransmitting form between the bugs and mammalians or various tissue cells in their hosts, and amastigotes (AMA) the living form in mammalian cells was separately cultured and then isolated. Each antiserum specific to EPI, TRP or AMA was elicited in rabbits. Three sensitive and accurate enzyme-linked immunosorbent assays (ELISA) for EPI, TRP and AMA were developed using the corresponding antiserum with β-D-galactosidase-labeled anti-rabbit IgG as the common second antibody and cell fragments of the corresponding parasites as antigens attached on Amino-Dylark solid-phase balls. The working range of each ELISA was $5\text{-}320\times10^3$ parasites/m*l* of EPI, and $1\text{-}100\times10^4$ for both TRP or AMA form parasites/m*l*. Accuracy of the assay results of each ELISA was demonstrated. It was found that ELISA for AMA was highly specific to AMA and contamination of various tissues of mice in assay samples gave no interference in the assay, proving that the direct assay of AMA present in various tissue cells was possible. Thus, mice were inoculated with 10^5 parasites of TRP, and time courses in the increased numbers of AMA in three tissues of the infected mice were assayed by the newly developed ELISA for AMA, which was possible to apply starting from the 5th day after the infection to the 18th day when all the infected mice were dead. Usual microscopic detection of the parasites in blood of the infected mice was possible at the 8th day. The mice were sacrificed and organs were collected. Thus, the first observations for time courses of infected parasites in heart ($2\text{-}400\times10^7$ parasites/g tissue), liver ($2\text{-}350\times10^6$ parasites/g tissue) and spleen ($8\text{-}80\times10^7$ parasites/g tissue) of the mice were successfully performed using the new tool.

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