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亜熱帯生育者，亜熱帯生まれ温帯移住者，温帯生育者の体格および体温の比較研究

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緒　　言

ヒトが高温環境に馴化すると，体温調節機能に適応的な変化があらわれ，一定高温負荷に対して体温の上昇度が減少し，より大きな高温負荷に耐えられるようになる（Dill *et al.*, 1938; Robinson *et al.*, 1941; Wyndham, 1967）。高温環境に曝露されたときの体温調節は，主として放熱の調節によってなされる。放熱量の増加は発汗がないか，もしあっても発汗量が少ない場合は主として皮膚血管の拡張による皮膚循環血流量の増加と皮膚温上昇によって，皮膚温と気温の差を大きくすることによってなされる（Robinson, 1949）。さらに高温負荷が大きくなると発汗が発現し，体温の蒸発調節が行われる。ヒトは発汗による体温調節能力が秀れており，高温馴化時には発汗反応に大きな変化があらわれるため，高温馴化時の発汗反応の適応的変化についての研究は数多いが，皮膚温に関する適応的変化の研究は少ない（Dill *et al.*, 1938; Kuno, 1956; Bass, 1963）。しかし，高温環境下での体温調節機能としては，皮膚温の調節能力は発汗反応と同様に重要である。高温馴化時にみられる適応的変化は，適応期間が短い場合と長期間に亙る場合では，その様相に差異があることが認められている。短期間の高温馴化時にみられる生理的反応は，暑熱負荷を中止すると比較的はやく失なわれることが知られている（Williams *et al.*, 1967）。しかし，長期間の高温馴化によって獲得された適応的変化は容易には消失しない（Hori *et al.*, 1976）。皮膚温は同じ環境下でも，夏は皮膚血管の緊張度が低下し，皮膚温が高く，冬は低くなる季節変動を示すが（季節生理班，1952; Herzman, 1956），高温環境に馴化した亜熱帯人が長期間温帯に生活するとき，皮膚温の調節機能がどのように変化するかを研究した報告はない。暑熱馴化が長期に及べば，体温調節の適応的変化の他に身体組織にも適応的変化があらわれ，体格や体構成が変化する。熱帯住民は痩せ型の体型をもつヒトが多く，体重当たりの体表面積が大きく，放熱に有利な体型をもつといわれている（Coon *et al.*, 1950）。また，高温季候下に生活すると他の組織より熱伝導率の低い（Keys and Brǒzek, 1953）皮下脂肪厚が減少して，体熱の放散を容易にする適応的変化があらわれるといわれている（堀ら，1974）．本土生育者の皮脂厚は冬に厚くなり，夏に薄くなる季節変動を示すといわれている（鈴木ら，1959）。生下時より高温環境によく馴化した亜熱帯生育者が本土の温帯季候下に生活すると，体型，体構成がどのような変化を示すかは興味深い。沖縄生育者が本土に移住した場合，本土の温帯季候が皮膚温の調節機能と，体格，体構成にどのような影響を及ぼすかを研究するために，沖縄生まれの本土移住者，および本土生育者の発汗準備状態での皮膚温の測定，および身体計測を行って，以前我々が沖縄本島で本研究と同じ条件下で測定した沖縄生育者の体温と身体計測結果を比較した（辻田ら，1978）。

実　験　方　法

沖縄生まれで本土在住期間が平均15.8年の成人

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男子12名，および本土生育者の成人男子11名の測定は西宮市で7月に行った。沖縄より本土に移住した群の対照群として，沖縄生育者の成人男子7名の測定を那覇市で7月に行った（辻田ら，1978）。皮脂厚は栄研式皮指厚計を用いて，皮膚接触面積 $1\,mm^2$ 当たり1gの一定圧で皮膚をつまんでから2秒後に測定を行い，数回の測定値の平均値をその部位の皮脂厚とした。皮脂厚の測定部位と平均皮脂厚算出に用いた加重平均係数は次の通りである（堀ら，1974）。

部　位		係数
上腕：	右腕背面，肩峰と肘頭の中間および右腕前面中央部の平均値	0.082
背部：	右肩甲骨下角	0.083
胸部：	右前腋下窩皺壁と右乳頭の中間	0.083
腹部：	右乳腺上，臍の高さ	0.081
腰部：	右肋骨下縁と右腸骨稜の中間	0.081
大腿：	右大腿中央，前面と背面の平均値	0.172

体脂肪含有率fは予知式（堀ら，1974）

$$f(\%)=28.9\times\frac{体表面積\,(m^2)\times平坂皮脂厚\,(mm)}{体重\,(kg)}+3.67$$

を用いて算出した。

体表面積の算出には，高比良の式（高比良，1925）を使用した。

皮膚温の測定は発汗のない状態での乾性放熱能力の指標としての比較が目的であるから，発汗準備状態での皮膚温を測定した。体温に対する運動，特異力学作用および日内変動をさけるために，測定日は被検者には激運動をさせず，空腹状態で午後3時より実験を開始した。被検者は水泳パンツだけを着用して，30 C，湿度70%の室内に30分間安静をとらせたのち，全身10カ所の皮膚温をパイロメーターを用いて測定した。舌下温は婦人体温計を用いて測定した。皮膚温の測定部位と平均皮膚温算出に用いた加重平均係数は次の通りである（Hori *et al.*, 1977）。

部　位		係数
額：	前額中央眉上2cm	0.098
胸：	乳腺と第4肋間の交点	0.083
腹：	乳腺上，臍の高さ	0.162
背：	肩甲骨下角直下	0.083
上腕：	三角筋中央点	0.082
前腕：	前腕内面中央線下から1/3	0.061
手：	手背中央線上から1/3	0.053
大腿：	大腿前面中央線上から1/2	0.172
下腿：	下腿後面中央線上から1/3	0.134
足：	足背中央線上から1/2	0.072

結　果

1．身体的特徴

沖縄生育者（O群），沖縄出身の本土住民（OM群），および本土生育者（M群）の身体計測値とOM群の本土滞在年数を表1に示した。OM群の身長，および体重の平均値はそれぞれ169.6 cmと62.8 kgで，M群の平均値170.2 cm，および64.3 kgよりわずかに小さかったが，O群の平均値166.6 cm，および58.6 kgよりかなり大きかった。OM群の胸囲，および上腕囲の平均値は90.4 cmと28.1 cmで，M群の平均値89.9 cm，および27.8 cmよりわずかに大きく，O群の平均値86.6 cm，および26.1 cmよりかなり大きかった。OM群の大腿囲の平均値は50.1 cmで，M群の平均値52.4 cmより小さく，O群の平均値49.6 cmよりは小さかった。これらの差のうち，O群の上腕囲が両群の平均値より有意に小さかったが，他の差は統計的には有意ではなかった。全体としてOM群はM群と比較してわずかに体格が小さい傾向を示したがほとんど差がなく，O群はOM群，およびM群と比較して全ての測定項目の平均値が小さく，かなり体格が小さいことが判る。

2．皮脂厚

皮脂厚の測定値は，表皮と真皮，および皮下脂肪層の厚さをそれぞれ二層ずつ含んでいるが，表皮と真皮の厚さは約1mmで個人差が少なく，皮脂厚の大小は皮下脂肪層の厚さに左右されるので，皮脂厚は皮下脂肪層の厚さを表わす指標として用いられる。皮脂厚の測定値を，部位別に表2に示した。測定された全ての部位でO群の平均値はOM群，およびM群の平均値より小さく，O群

Table 1 Characteristics of subjects

Group	Number	Age (yr)	Height (cm)	Weight (kg)	B.S.A. (m^2)	G.C. (cm)	G.A. (cm)	G.T. (cm)	Stay in main land (yr)
O	7	21.0 ±1.9	166.6 ±5.7	58.6 ±5.5	1.67 ±0.10	86.6 ±3.3	26.1* ±0.8	49.6 ±1.6	
OM	12	26.0 ±3.6	169.6 ±4.0	62.8 ±6.6	1.74 ±0.07	90.4 ±5.1	28.1 ±1.9	50.1 ±3.7	15.8 ±6.7
M	11	22.8 ±1.9	170.2 ±5.6	64.3 ±7.9	1.76 ±0.12	89.9 ±5.7	27.8 ±1.9	52.4 ±3.5	

O: Residents of Okinawa who were born and raised in Okinawa, OM: Residents of the Main land who were born and raised in Okinawa, M: Residents of the Main land who were born and raised in the Main land of Japan, B.S.A.: Body surface area, G.C.: Girth of chest, G.A.: Girth of upper arm, G.T.: Girth of thigh. Mean values are given with their standard deviations. * Significant difference between O group and other two groups at 5% level.

Table 2 Skinfold thickness of subjects

Group	Chest	Abdomen	Upper arm anterior	Upper arm posterior	Back	Waist	Thigh anterior	Thigh posterior
O	8.0 ±3.5	11.0 ±5.0	3.3 ±0.3	5.5 ±1.2	8.6 ±1.4	10.5 ±3.8	9.2 ±3.3	11.8 ±4.6
OM	11.1 ±6.9	15.8 ±7.7	3.7 ±1.2	7.6 ±3.1	10.8 ±4.0	16.5 ±6.8	11.7 ±4.2	13.9 ±5.5
M	10.6 ±6.9	14.3 ±8.3	4.0 ±1.7	7.9 ±3.3	9.8 ±2.4	18.4 ±9.4	9.4 ±4.1	13.9 ±6.5

O, OM, M: The same as in Table 1.
Mean values (in mm) are given with their standard deviations.

の皮下脂肪層の厚さは他の両群よりかなり薄かった。OM 群の平均値は胸部，腹部，背部および大腿前部で M 群の平均値よりわずかに大きく，上腕部，腰部ではわずかに小さかった。即ち，OM 群と M 群の皮脂厚はほとんど差がなかった。皮脂厚の最も厚い部位は O 群では大腿後面，OM 群と M 群では腰部であった。全体として O 群は他の両群に比較して軀幹部の皮脂厚がうすく，皮脂厚の部位による差が少ない傾向を示している。

3. 形態指数および栄養指数

身体計測値を用いて算出された Rohrer 指数，Brugsch 指数（比胸囲），体表面積と体重の比，体脂肪含有率，および平均皮脂厚の平均値と標準偏差を表3に示した。Rohrer 指数は身体充実度，または体型を示す指数であるが，OM 群の Rohrer 指数の平均値は 128.7で M 群の平均値 130.3より小さく，O 群の平均値126.5より大きい。OM 群の Brugsch 指数の平均値は53.3で，M 群の平均値53.7よりわずかに小さく，O 群の平均値 52.0よりかなり大きかった。体表面積と体重の比は，高温環境下での体温調節能力に関係する重要な指標であるが，この指標の平均値は OM 群が 278で M 群の平均値276よりわずかに大きく，O 群の平均値285よりかなり小さかった。体型を表わす Rohrer 指数，Brugsch 指数，体表面積と体重の比の OM 群の値はいずれも M 群と O 群の間にあって，M 群との差はわずかで，O 群との差はかなり大きかった。従って，OM 群の体型は O 群よりも，むしろ M 群に近いといえる。OM 群の体脂肪含有率の平均値は 13.3%で，M 群の平均値 12.8%，および O 群の平均値11.4%より大きく，平均皮脂厚も OM 群の平均値 12.2 mm

Table 3　Physical and nutritional indices

Group	Rohrer's index	Brugsch's index	B.S.A. (cm^2) / Weight (kg)	Body fat (%)	Mean S.T. (mm)
O	126.5 ±6.6	52.0 ±1.7	285 ±10	11.4 ±2.4	9.3 ±2.8
OM	128.7 ±13.0	53.3 ±2.9	278 ±14	13.3 ±3.2	12.2 ±4.5
M	130.3 ±12.3	53.7 ±3.7	276 ±16	12.8 ±3.2	11.7 ±4.6

B.S.A.: Body surface area,　Mean S.T.: Mean skinfold thickness,　O, OM, M: The same as in Table 1.
Mean values are given with their standard deviations.

は，M 群の平均値 11.7 mm，および O 群の平均値 9.3 mm より大きかった。体脂肪含有率と皮脂厚についても，OM 群と M 群の差はわずかであるが，OM 群と O 群の差はかなり大きい。

4．体温

口内温 (T_o)，測定部位別の皮膚温，平均皮膚温 ($\bar{T}_s$) および T_o-$\bar{T}_s$ gradient の平均値と標準偏差を表4に示した。OM 群の T_o の平均値は 37.1 C で M 群の平均値 37.2 C より 0.1 C 低く，O 群の平均値 37.0 C より 0.1 C 高かった。前額部の皮膚温は3群ともに最高値を示した。OM 群の前額部の平均値と M 群の平均値はともに 36.1 C で，O 群の平均値 35.9 C よりかなり高かった。軀幹部，即ち胸部，背部，腹部の皮膚温の平均値は，いずれも OM 群が最も高く，O 群が最も低かった。上肢の温度は3群で大差がなく，下肢の皮膚温は OM 群が最も低く，O 群が最も高かった。平均皮膚温は OM 群が 34.8 C で最も低く，M 群は 34.9 C, O 群は最も高く 35.0 C であった。T_o-$\bar{T}_s$ gradient の平均値は OM 群が

Table 4　Oral temperature and skin temperature

Group	O Mean	O S.D.	OM Mean	OM S.D.	M Mean	M S.D.
T_o	37.0*	0.2	37.1*	0.6	37.2	0.3
Forehead	35.9	0.3	36.1	0.8	36.1	0.6
Chest	34.4	1.0	35.3	0.6	34.8	0.7
Abdomen	34.5	0.9	35.0	0.7	34.5	0.8
Subscapular	35.0	1.0	35.1	0.5	35.1	0.6
Upper arm	35.2*	0.9	34.7	0.7	34.4*	0.7
Forearm	35.4	0.7	35.5	0.5	35.3	0.6
Hand	35.4	0.7	35.6	0.5	35.6	0.7
Thigh	34.8	0.9	34.4	0.7	34.7	0.9
Calf	35.0^{*a}	0.8	34.0^{*a}_{b}	0.8	34.8^{*b}	0.7
Foot	34.7	1.2	34.1	1.3	34.8	1.1
$\bar{T}_s$	35.0	0.7	34.8	0.6	34.9	0.6
T_o-$\bar{T}_s$	2.02	0.8	2.35	0.6	2.29	0.7

O, OM, M: The same as in Table 1.　T_o: Oral temperature,　$\bar{T}_s$: Mean skin temperature,
Mean values (in C) are given with their standard deviations.　*: Significant differences between two groups at 5% level.

2.35 C と最も大きく，M 群は 2.29 C でわずかに OM 群より小さく，O 群は 2.02 C で両群よりかなり小さかった。

考　　察

ヒトの体温調節機能は，季候の変化に応じて適応的な変化をうけることはよく知られているが (Robinson, 1949; Williams *et al.*, 1967)，異なる季候下に長期間生活すると体格，体型，身体構成も変化するといわれている (Lewis *et al.*, 1960)。亜熱帯の沖縄の季候は夏の高温多湿の期間が長く，気温の日内変動巾が小さい。冬は温暖で，沖縄住民は一年を通じて高温環境に曝露される機会が多い。一方，日本本土は温帯に属し，夏の高温期間が短く，冬は寒い。沖縄生育者と本土生育者の体温調節機能，体型，体構成を比較すると，沖縄生育者は高温環境下での体温調節機能が秀れており，皮下脂肪厚が薄く，体重当たりの体表面積が広い等の，高温環境下での体温調節に有利な体構成と体型をもっているといわれている (Hori *et al.*, 1977)。本研究の被検者の OM 群は，沖縄に生まれて幼少年期を沖縄で過ごした後，本土に移住して本土での在住期間が平均15.8年と長く，O 群は本土での生活経験がないので，OM 群と O 群の差は，少年期以後の OM 群の温帯季候下での生活によって，もたらされたものと考えられる。OM 群と M 群の差は，OM 群が幼少年期に熱帯季候下で生活したことによる差とみなしうる。OM 群は O 群と比較して，身長，体重，Rohrer 指数，Brugsch 指数の平均値がかなり大きく，M 群と比較するとわずかに小さい。低温季候下に生活すると体格が大きくなり，体重が重くなって，身長に比して軀幹部が大きくなって，体重も増加するといわれている (Coon *et al.*, 1950)。従って OM 群の体格や体型が，O 群よりむしろ M 群に近いことは，OM 群が温帯で幼少年期以後を過し，寒冷季候下で長く生活した影響が，幼少年期に生育した熱帯季候の影響より大きいものと推定される。歩行や走行等の体重の移動を伴う運動を行うときの代謝量は，体表面積よりむしろ体重に比例するが，放熱量は体表面積に比例する。従って体重当たりの体表面積の比が大きい体型のヒトは，高温環境下で体温の調節能力が大きい。この値の O 群の平均値は OM 群，M 群より大きく，OM 群と M 群の差はわずかである。以上より O 群は M 群より高温環境に適応した体型をもっているが，OM 群は M 群とほとんど同じ体型であると結論できる。しかし，季候以外にも栄養状態や身体運動を含めた生活様式も，身体の発育に影響をおよぼすことが知られているので，OM 群と O 群の身体的差異は季候以外の因子の関与もあると考えられる。

表 2 に示されたように，皮下脂肪厚の平均値は測定された全ての部位で，O 群は他の両群よりかなり小さく，OM 群の平均皮脂厚の平均値は M 群の平均値よりわずかに小さい。脂肪組織の熱伝導率は，非脂肪組織の熱伝導率より低く，皮下脂肪層の厚さが薄くなると体熱の放散が容易に行われる (Keys *et al.*, 1953)。従って O 群の体熱放散に関しては，OM 群および M 群より有利であると推定される。30 C の環境温は，安静状態のヒトが発汗を発現させずに，皮膚血管の調節で体熱の平衡を維持できる最高外気温である。このときのヒトの状態は，外気温が上昇するとすぐに温熱性発汗が発現するので，発汗準備状態といわれている。皮膚面からの放熱量のうち，伝導，対流および輻射の機転による乾性放熱量は皮膚温と外気温の差に比例するので，放熱量は皮膚温の高い方が大きく，皮膚温の高さは放熱能力を示すことになる。

表 4 に示したように，O 群の平均皮膚温は OM 群，および M 群より高く，このことは O 群が他の両群より放熱量が大きいことを示唆する。局所の皮膚温を比較すると O 群は OM 群，M 群と比較して四肢，ことに下肢の皮膚温が高い。四肢の表面積は体表面積の60%をしめ，この部位の皮膚温の上昇が大きいことは，皮膚からの放熱量を大きくする。このことは O 群は OM 群，M 群に比し，四肢の皮膚血管の拡張度が強く，四肢の皮膚循環血流量が増加して四肢の皮膚温が高くなったものと考えられる (Hertzman, 1959; Rothman,

1954)。T_o-$\bar{T}_s$ gradient は安静状態で代謝量にほとんど差がなく体温の変化しないときは，身体の熱貫流率と逆比例の関係にある。O 群の T_o-$\bar{T}_s$ gradient の平均値は 2.02 C で OM 群の平均値 2.35 C, および M 群の 2.29 C よりかなり小さい。従って O 群は，他の両群より高い熱貫流率をもっていることを示す。この O 群の大きな身体熱貫流率は，高温馴化による血液量の増加と皮膚血流量の増加による高皮膚温の維持能力の向上によりもたらされたものと推定される (Bass *et al.*, 1936; Hertzman, 1959)。OM 群と M 群の身体の熱貫流率はわずかである。即ち，生下時より成人まで亜熱帯季候下に生活したヒトは高い熱貫流率を保ち，皮膚温を高く維持する能力を獲得して，秀れた乾性放熱能をもつことができるが，幼少年期に亜熱帯季候下に生活し，少年期以後を温帯季候下で長期間生活したヒトは，生下時より温帯に生活したヒトと同程度の熱貫流率と乾性放熱能を持っていると結論できる。

要　　約

沖縄生育者の成人男子7名 (O 群)，沖縄生まれで本土在住期間が長い成人男子12名 (OM 群)，および本土生育者11名 (M 群) の身体計測と 30 C, 湿度70%の室内にて安静座位における口内温と全身10カ所の皮膚温を測定して次のような結果を得た。

1. OM 群は体格，体型ともに M 群とほとんど差がなく，O 群より身長は高く，体重が重く，胸囲，上腕囲，大腿囲も大きかった。OM 群の形態指数は M 群との差は少なく，O 群と比較すると Rohrer 指数，比胸囲が大きく，体重当たりの体表面積比が小さかった。

2. OM 群の皮下脂肪厚，および体脂肪含有率は M 群とほとんど差がなく，O 群と比較すると皮下脂肪厚は薄く，体脂肪含有率が少なかった。

3. 30 C における OM 群の口内温の平均値は 37.1 C で M 群より 0.1 C 低く，O 群より 0.1 C 高かった。平均皮膚温は OM 群の平均値は 34.8 C で M 群より 0.1 C 低く，O 群より 0.2 C 低かった。口内温と平均皮膚温の差は OM 群では2.35 C で M 群の 2.29 C よりわずかに大きく，O 群の 2.02 C よりかなり大きかった。

4. OM 群の体格，体型，乾性放熱能力，身体の熱貫流率は M 群とほとんど差がなく，O 群は高温環境に馴化した熱帯人に近い体格，体型を示し，他の両群と比較して秀れた乾性放熱能力を持ち，高い身体の熱貫流率をもつことが判った。

5. 幼少年期に亜熱帯に生活した後，温帯に移住して長期間温帯季候下で生活すると体格，体型および高温環境下での耐熱放熱能力は亜熱帯生育者よりむしろ温帯生育者のそれに近くなる。

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COMPARISON OF PHYSIQUE AND BODY TEMPERATURE AMONG SUBTROPICAL NATIVES, IMMIGRANTS OF SUBTROPICAL NATIVES TO TEMPERATE ZONE AND TEMPERATE NATIVES

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Anthropometrical measurements and measurements of body temperature were made in July in Naha (Okinawa, subtropical zone) on 7 young male Japanese who were born and reared in Okinawa (group O), and in July in Nishinomiya on 12 young male residents of Main Islands of Japan (temperate zone) born and reared in Okinawa (group OM) and 11 young male residents of Main Islands born and reared in Main Islands (group M). The mean value of duration of residence in Main Islands of subjects in group OM was 15.8 years. Skin temperatures at 10 sites and oral temperature of seated subjects, wearing short only, were measured in a climatic chamber maintained at 30 C and 70 per cent relative humidity after sitting in a chair at rest in the climatic chamber.

Group O showed shorter height, lighter body weight, larger girths of chest, upper arm and thigh than group OM and M. Group O showed smaller mean values of Rohrer's index and Brugsch's index and greater mean value of the ratio of body surface area to body weight. Group O showed thinner skinfold thickness and smaller percentage of body fat content than other two groups. Differences of anthropometrical measurement between group OM and group M were much less when compared with those between group O and other two groups. Mean values of oral temperature for group O, OM and M were 37.0 C, 37.1 C and 37.2 C respectively. The mean values of mean skin temperatures for group O, OM and M were 35.0 C, 34.8 C and 34.9 C respectively. Group O showed considerably smaller value of $T_o - \bar{T}_s$ gradient (2.02 C) than group OM (2.35 C) and group M (2.29 C). These results indicate that physical characteristics of subtropical natives have changed and that difference of physical characteristics between subtropical natives and temperate natives and capacity of maintaining higher skin temperature and higher conductive-convective heat transfer coefficient observed in subtropical natives were reduced by long residence in a temperate zone.

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AN ATTEMPT FOR SHORT-TERM CULTIVATION OF *TRYPANOSOMA GAMBIENSE* IN COEXISTENCE WITH THE MOUSE L-CELL (AT 37 C)

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Abstract: It was attempted to cultivate *Trypanosoma gambiense* (Wellcome strain), maintained by mouse-passages for years, in coexistence with the mouse L-cells *in vitro* at 37 C. In the system of culture the population of the cultured trypanosomes increased 3-fold in 24 hours, then decreased. The fact that the doubling time of trypanosomes was longer in the culture system than in an infected mouse seemed to be related to the suppressed division of parasites. It was observed that the final stage of parasite division, that is, the cytoplasmic separation into 2 individuals was much prolonged in culture, although the kinetoplastic, nucleal and flagellar duplication was carried through without suppression. In the control without the L-cells, no multiplication of trypanosomes took place. Infectivity of the cultured trypanosomes to mice did not change throughout the course of culture, at least until the time of the maximum growth of parasites. The sub-culture of trypanosomes was not successful.

INTRODUCTION

The *in vitro* cultivation of the trypanosomes belonging to the *brucei* group is generally considered to be difficult to establish. Many studies on the matter have been attempted since Novy and McNeal (1903, 1904) reported some success in cultivation of *Trypanosoma brucei* in a diphasic blood-agar medium called NN medium. According to reports so far published, results of the cultivation of the trypanosomes varied greatly depending on the species or strains of the parasites, and the temperature applied to the experiments.

For the cultivation of the African sleeping sickness trypanosomes for the purpose of diagnosis, Weinman (1944, 1946) presented a culture method using a semi-solid medium which consisted of nutrient agar, human plasma and haemoglobin. Tobie *et al.* (1950) reported a diphasic nutrient blood-agar medium, using rabbit blood instead of human one. In this medium *T. gambiense* and *T. rhodesiense* transferred from laboratory animals were found to develop only to the proventricular forms losing infectivity to laboratory animals as usually observed in cultures tried at the temperature below 37 C. For a valuable diagnostic procedure, a haemoculture

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technique was reported on *T. gambiense* by Neujean and Evens (1952), and Weinman (1960) described a reliable culture method for diagnosis and isolation of *T. gambiense* and *T. rhodesiense*. In a defined or semi-defined medium, *T. gambiense* (Nicoli, 1961), *T. rhodesiense* and *T. brucei* (Cross and Manning, 1973) were found to grow at the culture temperature of 25–28 C. Although many attempts were done as stated above, at the body temperature of mammals it has not been achieved to cultivate the blood stream forms of the *brucei* group trypanosomes. In this connection, Williamson and Rollo in 1952 observed survival in undiminished numbers of *T. rhodesiense*, for about 4 hours at 37 C in serum-free synthetic medium.

In recent years, the tissue culture methods were employed in the cultivation of *T. brucei* by several workers. Le Page (1967) reported that the mouse L-cells in NCTC 109 medium with 10 per cent calf serum could be used in cultivation of *T. brucei* at 37 C. Later, Eagle's MEM medium with 20 per cent calf serum was employed as the basal medium by Hawking (1971). It was reported in those studies that the trypanosomes inoculated into the media were found to multiply 2- to 5-fold within 24 hours of cultivation, but subculture was not successful. Hirumi *et al.* (1977 a, b) first reported on the successful cultivation of the blood stream forms of *T. brucei* at 37 C with use of the tissue culture system composed of the bovine fibroblast-like cell and HEPES-buffered RPMI 1640 with 20 per cent heat-inactivated fetal bovine serum.

The present study was an attempt of cultivation of *T. gambiense* maintained by mouse-passages for years, using tissue cultures of the mouse L-cell. As the result, no establishment of the culture has been achieved, but some results could be obtained of the multiplication, the morphological characters and the infectivity to mice on the cultured parasites during the short-term cultivation.

Materials

Trypanosoma gambiense employed in this study was the Wellcome strain which had been maintained by serial passages through mice for years in our laboratory. Male ddO mice, 6–7 weeks old, were used as the doners of the blood stream forms of *T. gambiense* and also brought to the infectivity test of cultured trypanosomes.

The mouse L-cell line was kindly supplied by the Department of Pathology in this institute. The tissue culture medium for cultivation of *T. gambiense* with the mouse L-cells was Eagle's MEM medium containing heat-inactivated calf serum at 10 per cent and antibiotics (100 U/m*l* of penicillin G and 100 μg/m*l* of streptomycin sulfate).

In addition the miscellaneous experiments were performed as follows. As the basal medium, RPMI 1640 or TCM 199 medium was tried in the place of Eagle's MEM medium. As the serum component, heat-inactivated rabbit, rat, mouse or fetal calf serum was also tried in the same concentration as that of calf serum. For the mouse L-cell, another line of mouse fibroblast cell was tested.

Experimental Methods

Collection of trypanosomes: About 48 hours after the intraperitoneal inoculation of 10^5 trypanosomes into each mouse, more than 10^8 parasites per m*l* could be found in the peripheral blood of the mice. Blood was drawn from the heart of the infected mice with a syringe containing Alsever solution of almost equal volume to mouse blood to be taken, then transferred into a mixture of Alsever solution (10 m*l*) and culture medium (20 m*l*) in a centrifuge tube. The contents were centrifuged at 200 *g* for 5 min to sediment the blood cells and the supernatant was again centrifuged at 1,000 *g* for 10 min to collect trypanosomes. The sediment was resuspended in the culture medium and the parasite population was adjusted to 7.5×10^6/m*l*. All the procedure to prepare the final parasite suspension was carried out at 4 C so as to keep the fixed condition.

Preparation of L-cell cultures: Cultures of the mouse L-cell were prepared in TD-40 flat culture bottles. The culture medium was the same as that for parasite culture described in the chapter of materials and 15 m*l* of the medium was used a bottle. Each culture bottle was plugged with a spongy silicone stopper and incubated at 37 C in air mixed with CO_2 at 5 per cent. These methods and condition were also applied to the cultivation of trypanosomes. L-cell monolayers were serially subcultured at 5- to 7-day intervals. For the cultivation of trypanosomes, 2×10^6 L-cells were inoculated into a fresh culture medium 4 days before the trypanosome inoculation, according to the results of the preliminary experiment that a confluent monolayer was formed from 2×10^6 L-cells within 3 days. The cultivation of trypanosomes was mainly tried in coexistence with a monolayer of the L-cells. The cultivation, however, was sometimes tried with a smaller number of the L-cells than in a monolayer, at the growing stage of separate colonies, in order to examine the influence of the growth condition of the L-cells. In preparation for this trial, 1×10^5 L-cells were inoculated into a fresh culture medium 4 days before trypanosome inoculation.

Inoculation of *T. gambiense* into the mouse L-cell cultures: The medium of the mouse L-cell cultures was pipetted off, 14 m*l* of the fresh culture medium was added into each culture 2 hours before the inoculation of parasites. One m*l* of trypanosome suspension (7.5×10^6 trypanosomes/m*l*) prepared from infected mice was introduced into each of the tissue cultures. Thus, the parasite population became 5×10^5/m*l* at the initiation of culture.

Examination of cultured trypanosomes: Usually every 4 to 8 hours, a small amount of the liquid portion of culture was sampled with small pipettes and all living trypanosomes and dividing forms were counted separately with haemocytometer under a phase-contrast microscope. From the results, the growth curve of cultured trypanosomes and also the ratio of dividing forms to all living trypanosomes obtained, respectively. At the same time Giemsa-stained smears were prepared for classification of the parasites into the following 4 types of growing stages: 1) trypanosomes with one kinetoplast and one nucleus, (1K, 1N)-type; 2) 2 kinetoplasts and one nucleus, (2K, 1N)-type; 3) 2 kinetoplasts and 2 nuclei; (2K, 2N)- type; 4) no

kinetoplast and one nucleus, (0K, 1N)-type. The percentage of each type was calculated from 1,000 parasites counted. To examine infectivity of cultured trypanosomes, 10^3 or 50 parasites in 0.5 m*l* of saline, taken from cultures at 12-hour intervals, were inoculated intraperitoneally into each of 4 mice, then the mice were subjected to examination of trypanosomes in the peripheral blood. A drop of blood from the tail of infected mice was placed on a slide glass and covered with a cover slip (24 mm $\times$ 24 mm), then examined for trypanosomes at $\times$400 magnification with a microscope.

Results

The growth curves of trypanosomes which were inoculated into the L-cell culture under different conditions were shown in Figure 1. On the L-cell layer which had been grown enough to cover the bottom of a culture bottle completely, trypanosomes were found to start multiply without lag phase and the number of trypanosomes were became 2-fold between 14 and 16 hours of culture and 3-fold within 24 hours, then decreased. On the other hand, over the L-cell layer which was just at the stage of separate colony formation, covering almost 1/4 area of the bottom of a culture bottle, the growth period of trypanosomes was usually prolonged and the number of trypanosomes decreased gradually, although the multiplication rate was lower. In the culture of trypanosomes without using L-cells, trypanosomes could not multiply and decreased within 12 hours.

Growth activity of trypanosomes in culture varied greatly due to the phase of parasitaemia in an infected mouse at the time of bleeding for parasite collection. Trypanosomes collected from an infected mouse with the maximum parasitaemia of over 1×10^9 parasites per m*l* of blood, showed multiplication only during such a

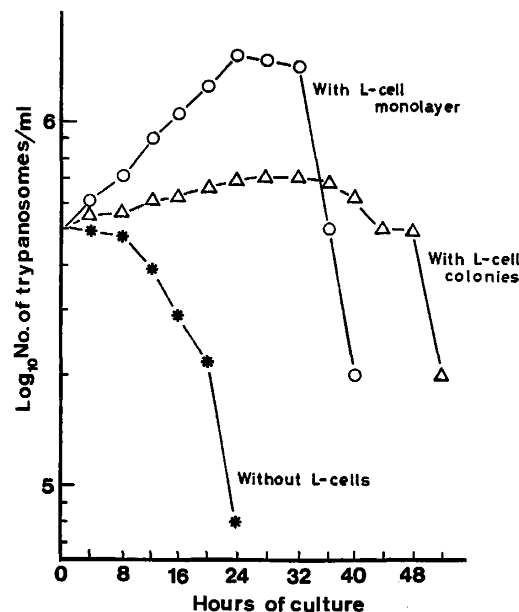

Figure 1 Growth curves of trypanosomes in culture.

short period as 6 to 8 hours. Therefore, parasites at the peak of parasitaemia could be regarded as inadequate materials for inoculation into this culture system.

The dividing forms of living trypanosomes shown in Figure 2 as percentage along the course of culture time. In this study the dividing form indicated a trypanosome at the stage of incomplete separation into 2 individuals but showing the partial separation of the cytoplasm in the anterior part. The proportion of dividing forms of cultured trypanosomes with the L-cells were observed to increase for 16 hours of cultivation, reaching almost 1/3 of the whole population of cultured trypanosomes, then decreased. Under the condition that trypanosomes could not multiply in the culture without the L-cells, the proportion of the dividing forms remained at the initial level for 24 hours of observation.

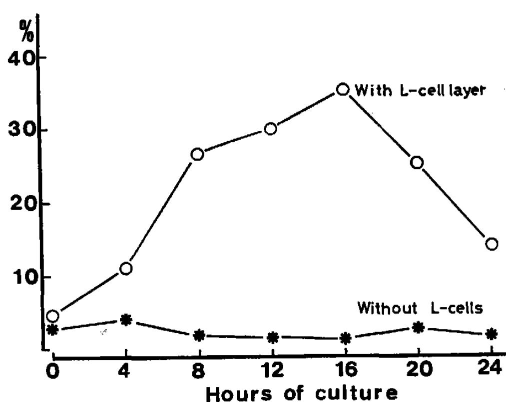

Figure 2 Proportion of dividing forms.
The proportion was determined by calculating the ratio of the dividing forms to all living parasites from the numbers counted under a phase-contrast microscope.

Trypanosomes in culture were counted, differentiating into 4 types of growing stages on Giemsa-stained smears. As shown in Figure 3, the ratio of (1K, 1N)-type trypanosomes apparently shifted to that of (2K, 2N)-type, passing through the stage of (2K, 1N)-type in accordance with culture time. In the culture system with the L-cells the proportion of (2K, 2N)-type trypanosomes which were thought to be at the most advanced stage of division reached the highest level, 32 per cent in 20 hours of cultivation, then became lower. On the other hand, in the culture without L-cells, in which no multiplication of trypanosomes took place, the portion of (2K, 2N)-type increased to occupy 1/3 portion of the total population in 8 to 12 hours of cultivation time. From the view of the kinetics of trypanosome division, it might be interesting that the maximum portion of the dividing forms showed similar value to that of the (2K, 2N)-type in the culture system with L-cells. On the other hand, in the culture system without L-cells, the proportion of the dividing forms stayed at a very low value throughout the culture period, although the (2K, 2N)-type showed an increase in proportion. It was understood from this result that the cytoplasmic division of the (2K, 2N)-type parasites would be highly suppressed in the culture system without L-cells. Furthermore, in the absence of L-cells, it was impossible to

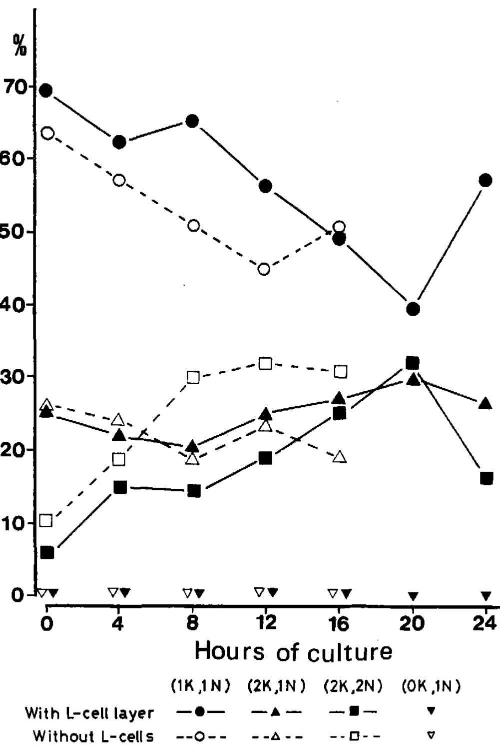

Figure 3 Proportion of 4 types classified by growing stages. The percentage was determined by observation of Giemsa-stained smears. The proportion of the (0K, 1N)-type did not rise above 0.2 per cent.

classify into 4 types in smeared preparations after 16 hours of culture, because of the rapid increase of degenerated parasites.

The infectivity test to mice of the cultured trypanosomes with L-cells, taken every 12 hours from the culture, was undertaken with 10^3 trypanosomes per mouse as inoculum size. The infectivity was measured by the prepatent period during which no parasite could be found in the peripheral blood of mice through 20 fields at $\times 400$ magnification and the days after inoculation until death of mice. It was observed that trypanosomes within 36 hours of cultivation did not show any different feature of infectivity to mice in comparison with the control trypanosomes inoculated

Table 1 Infectivity of cultured trypanosomes to mice

Inoculum size	A: 10^3				B: 50	
Culture time (hours)	0 (Control)	12	24	36	0 (Control)	52
Mice	a b c d	a b c d	a b c d	a b c d	a b c d	a b c d
Prepatent period (days)	2 2 2 2	2 2 2 2	2 2 2 2	2 2 2 2	3 3 3 3	3 3 3 3
Days until death	4 5 5 5	5 5 5 4	5 6 5 5	5 5 5 5	6 6 6 6	7 6 6 6

A: cultured with L-cell monolayer B: cultured with L-cell colonies

at time zero. Furthermore, even with as few as 50 trypanosomes in the 52-hour cultivation no lower infectivity to mice could be found (Table 1).

The subinoculation into fresh tissue culture with a part of the culture suspension or cultured trypanosomes collected by centrifugation was tried at different phases of the culture course, but the transferred parasites did not multiply, showing rapid decrease in number.

In the miscellaneous experiments, firstly RPMI 1640 and TCM 199 as the basal medium were examined for the supporting efficacy upon parasite growth, respectively. Secondly, heat-inactivated rabbit, rat, mouse and fetal calf serum was also examined respectively, in place of inactivated calf serum. Finally, another mouse fibroblast cell line was tried instead of the L-cell. All these attempts gave the similar results as described hitherto.

Discussion

Establishment of the *in vitro* cultivation of the blood stream forms of the *brucei* group trypanosomes has been expected for a long time. Hirumi *et al.* first in recent years reported the successful cultivation of *T. brucei*, applying the tissue culture method.

From the results obtained in this study, the presence of tissue culture cells was thought to be indispensable to support the multiplication and survivability of *T. gambiense in vitro*, though within limitation. Although many attempts were done, the doubling time of trypanosomes in culture appeared to be much prolonged as compared with that in infected mice. From these facts, this culture system might be lacking some components which would be essential for growth of trypanosomes. On the basis of this consideration, the necessary components were probably carried over into the culture system by trypanosomes when inoculated from infected mice. After the consumption or dilution of these components by trypanosomes in culture, their multiplication would be ceased and the number of them would decrease. This would be reasonably thought from the fact that there was no lag phase until the beginning of multiplication of trypanosomes. Those related facts would also give the reason why the subculture of trypanosomes was impossible.

The accumulation of the dividing forms and of the (2K, 2N)-type parasites in culture was not assumed to indicate the inhibition of the division of kinetoplast, nucleus or flagellum, but the cytoplasmic division was probably suppressed by some unknown factors, although other factors to promote the growth and survival of trypanosomes would be contained in the culture medium and also supplied by L-cells.

This cultivation was carried out at 37 C and trypanosomes growing in culture did not show any loss of infectivity to mice. Therefore, *T. gambiense* in this culture could be regarded to maintain the characters of the blood stream forms of the parasites, although more detailed examinations should be necessary to determine it. From these standpoints, in a shorter period less than 24 hours, this cultivation system for *T. gambiense* was thought to be effectively applicable to some *in vitro* experiments of this parasite strain.

The outline of this paper was reported at the 32nd West Japan Regional Meeting of the Japanese Society of Parasitology in Kurashiki City, November 11, 1976.

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Trypanosoma gambiense の 37 C における短期間培養マウス L-細胞を共存させての試み

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マウスにより長期にわたり継代維持されてきた *Trypanosoma gambiense* (T. g.), Wellcome 株を *in vitro* 37 C でマウス L-細胞と共存させて培養することを試みた。この培養系において T. g. は lag phase なしに増殖し始め，24時間で T. g. 数は3倍になり，以後減少していった。T. g. 数が2倍になるまで12-14時間を要したので，この系での世代時間は，感染マウス内での場合（約5時間）にくらべ非常に長くなっている。培養された T. g. の観察結果から T. g. の分裂過程において形態上キネトプラスト，鞭毛および核は倍化しているにもかかわらず，それに続いて起こるべき虫体の縦裂そして2原虫への分離の段階において時間を要していることがわかった。つまりこの分裂が部分的に抑制されているために増殖が遅くなったと考えられる。対照として L-細胞の共存なしで，培養された T. g. では，やはりキネトプラスト，鞭毛および核の倍化は起こっているにもかかわらず，増殖が認められなかったのでこの場合にはより高度に T. g. の分裂が抑制されていると考えられる。

従って具体的な要因は不明であるが L-細胞の存在は T. g. の生存，増殖に有用であるといえる。この系で培養された T. g. は少なくとも T. g. 数が減少し始めるまでの間，培養開始と同じ程度にマウスに対する感染能を保持していた。しかしながらこの系において T. g. を継代することはできなかった。以上のことから，本培養系では T. g. を培養維持することはできないが，原虫数が減少し始めるまでの時間，つまり24時間以内に限定すれば，本原虫を用いた他の実験にこの培養法を応用することができると考えられる。

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DEVELOPMENT OF *BRUGIA PAHANGI* MICROFILARIAE FROM JIRD PERITONEAL CAVITY IN *AEDES AEGYPTI*

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Abstract: A new apparatus for feeding mosquitoes is described. It is constructed without special technique and easy of handling. The usefulness of the apparatus is demonstrated by a report on investigations into the effects of nucleotides on mosquito gorging and the development of the microfilariae from the jird peritoneal cavity and from the dog venous blood in *Aedes aegypti*. As a phagostimulant 10^{-3}M ATP is the most effective of the nucleotides tested. The microfilariae from the jird peritoneal cavity have been proved to reach stage III (i.e. infective stage) as those from the dog, although the rate is lower in the former. It is also proved that the microfilariae develop to infective larvae in the mosquitoes which have not taken blood, serum or protein meals.

INTRODUCTION

Brugia pahangi, whose normal hosts are cats and dogs (Buckley and Edeson, 1956; Laing *et al.*, 1960; Schacher, 1962 a, b), was successfully transmitted to the jird (*Meriones unguiculatus*) with subcutaneous inoculation of infective larvae (Ash and Riley, 1970; Ash, 1973). When the infective larvae were inoculated into the peritoneal cavity of the jird, adults and microfilariae were recovered from the peritoneal cavity (McCall *et al.*, 1973). The adults and microfilariae localized in the peritoneal cavity seem to follow an aberrant mode of development, although the ease of recovery of worms from the small rodents may serve for the studies on the biology, biochemistry, immunology and host-parasite relationships of filariasis. A search in the literature failed to reveal any report on the development of the microfilariae from the peritoneal cavity in mosquitoes.

There have been described several methods of feeding hematophagous arthropods artificially through natural and artificial membranes. Most of the devices have utilized a flat membrane extended in a horizontal plane. The devices with flat membranes have been used for mosquitoes by previous authors (St. John *et al.*, 1930; Woke, 1937; Bishop and Gilchrist, 1946; Eyles, 1952; Kartman, 1953; Bar-Zeev and Smith, 1959; Collins, 1963; Collins *et al.*, 1964; Rutledge *et al.*, 1964; Behin, 1967). In most of such devices it is difficult to maintain the solution offered to the arthropods at a certain temperature suitable for feeding. On the other hand, Pipkin and Connor (1968) have reported a device with a pouch-like membrane of rubber, sheep caecum or pig intestine sheathing a test tube with rounded bottom and also Kitaoka

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and Morii (1970) a feeder with a membrane of chicken crop covering a round bottom tube, which provide sufficient surface areas of the membranes for mass feeding of colonized vectors. In the latter devices the temperature of feeding solution can be maintained with ease, although relatively large quantity of feeding solution has to be filled in the space between the membrane and the tube.

Hosoi (1959) has examined a variety of chemicals dissolved in buffered saline offering to *Culex pipiens pallens* singly confined in a glass tube with a forcible feeding technique and reported that some nucleotides stimulate the mosquito into gorging with a blood meal, which is dispatched only to the stomach. Rutledge *et al.* (1964) found that adenosine triphosphate is a phagostimulant to some mosquitoes.

As the vector mosquitoes for *Brugia pahangi*, *Armigeres subalbatus* and/or *Aedes togoi* were used by previous authors (Ash and Riley, 1970; Ash, 1973; Kan and Ho, 1973; Nakajima *et al.*, 1976). In the present studies Liverpool strain of *Aedes aegypti* has been used because of ease of maintenance and feeding as well as susceptibility to *Brugia pahangi* infection.

This paper describes a new apparatus designed for feeding microfilariae to mosquitoes, the phagostimulant effect of nucleotides on *Aedes aegypti* and the development of microfilariae from the jird peritoneal cavity in the mosquito in comparison with those from the dog venous blood. It also discusses the effect of protein meals for mosquitoes on the development of larvae.

Materials and Methods

I. Feeding apparatus, solutions and mosquitoes

The feeding apparatus consisted of a glass jar about 8 cm in height with a flat bottom about 4 cm in diameter and a rubber stopper inserted with two cannulae. The cannulae were connected to a water pump with two flexible polyvinyl tubes. The inside of the jar was circulated with water from a thermostatically controlled water bath whose temperature was maintained at 38 C.

Mice were sacrificed under ether anesthesia. The fur on the skin was clipped off as closely as possible with a pair of electric clippers. The skin was then removed from the carcass and the subcutaneous and fatty tissues were eliminated. The skin was washed with saline and stored in a refrigerator or a freezer prior to use. When needed, the skin membrane was washed with saline and stretched, the subcutaneous side inward, over the bottom of the jar, to which it was held by a rubber band. A Pasteur pipette was inserted between the membrane and the side of the jar. Commercially available, salted outermost layer of sheep intestine and prophylactic rubber condoms were also tried as feeding membrane. Their use was eventually discontinued, because the former tended to be leaky after freshening and mosquitoes had difficulty in piercing the latter.

The feeding solutions tested were physiological saline, Dulbecco's phosphate-buffered saline without calcium or magnesium (PBS) and bovine serum. The bovine serum was light red in color due to hemolysis. The nucleotides used as phagostimulants were adenosine 5′-monophosphoric acid (from equine muscle,

Type V, Sigma Chemical Co.) (AMP), adenosine 5'-diphosphate (from equine muscle, Sodium salt, Grade I, Sigma Chemical Co.) (ADP) and adenosine 5'-triphosphate (disodium salt, P-L Biochemicals Inc.) (ATP) dissolved in the final concentration of 10^{-4} M or that of 10^{-3} M. The nucleotides were added to the cold solutions just prior to the experiment and placed in an ice bath until feeding.

Controls were the solutions without nucleotides as well as dog serum and

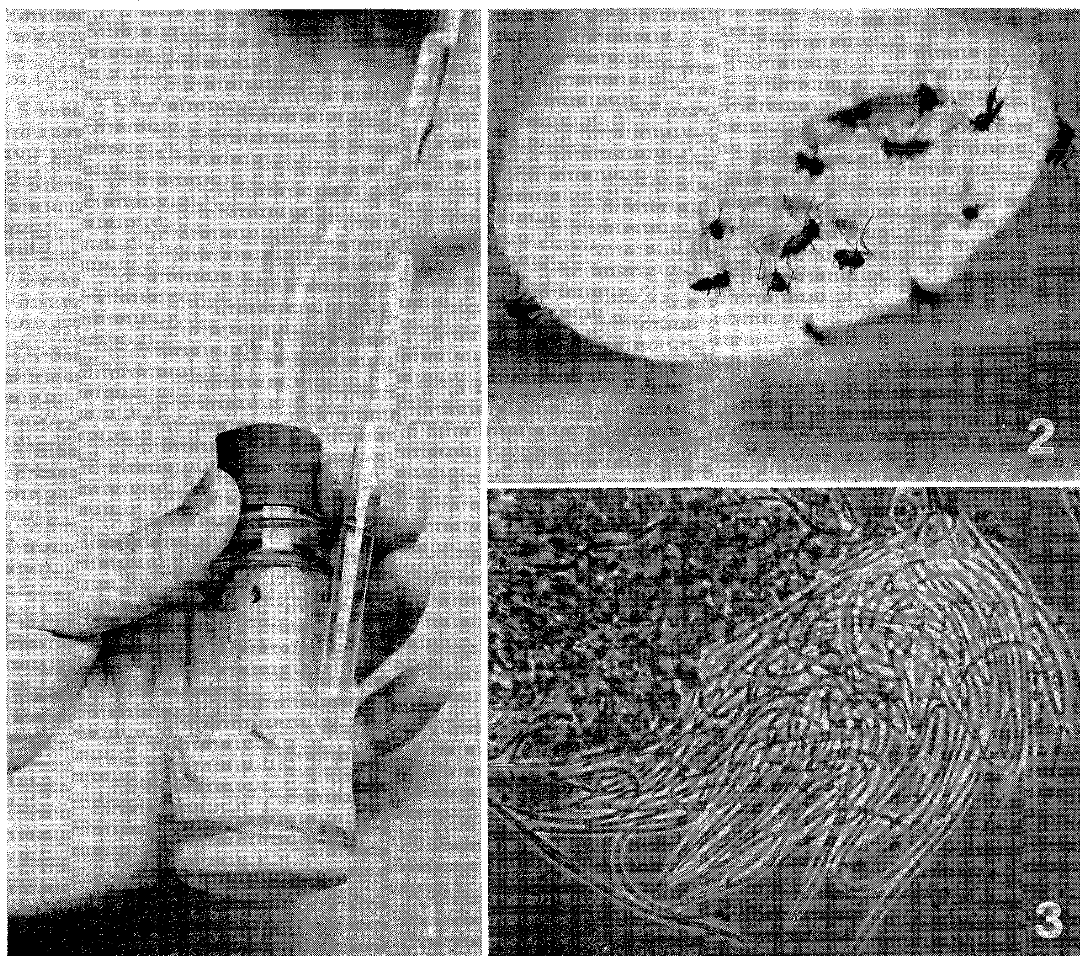

Figure 1 Feeding apparatus for mosquitoes.
A skin membrane is stretched over the flat bottom of the glass jar. Feeding solution is placed into the space between the jar bottom and the membrane through Pasteur pipette. Inside of the jar was circulated with water from a temperature-controlled water bath through a water pump, two flexible tubes and two cannulae.

Figure 2 Mosquitoes piercing the membrane of the apparatus hung in the cage and gorging feeding solution.

Figure 3 Phase-contrast microphotograph of stomach contents of *Aedes aegypti* dissected immediately after artificial feeding of microfilariae.
As shown in the figure, overcrowding of microfilariae in the stomach was occasionally observed in the group of mosquitoes offered the microfilariae from jird peritoneal cavity at the density of 1,000 to 1,500 per 0.03 m*l* of PBS.

defibrinated rabbit blood. In some experiments a drop of Indian ink or a drop of one per cent patent blue solution was added to each 2 to 3 m*l* solution for easy identification of the solutions in the alimentary tract of mosquitoes.

The feeding solution, 2 to 6 m*l*, was placed into the space between the jar bottom and the skin membrane through the Pasteur pipette, which was then removed (Figure 1). The side of the jar was covered by a polystyrene sheet.

The mosquitoes used were *Aedes aegypti* (Liverpool strain) which were maintained in our laboratory. For feeding trials 28 to 100 female mosquitoes were put into a cage about 40 cm long, 30 cm wide and 30 cm high. Age range was 5 to 7 days from the day of emergence. The diet, 2 per cent sucrose solution, was removed 24 hours before the trials.

The jar was hung in the cage through the top hole of the cage (Figure 2). The ambient temperature was 17 to 25.5 C. The cage was covered up with a sheet of black cloth. The test period for each cage was 30 minutes. Immediately after the feeding, the mosquitoes were anesthetized with ether and dissected under an operating microscope.

II. Infection of *Aedes aegypti* with *Brugia pahangi* microfilariae

Microfilariae were recovered from the peritoneal cavity of the infected jirds (*Meriones unguiculatus*) which had been inoculated intraperitoneally with the infective (i.e. stage III) larvae of *B. pahangi*. After the injection of 10 m*l* of PBS into the peritoneal cavity, the syringe was removed and the $18G \times 1\frac{1}{2}''$ (1.2×38 mm) needle was left to recover the injected fluid containing microfilariae. The fluid dripping through the needle was collected, mixed with cold PBS and centrifuged for 5 min at 6,000 rpm, 0 C. The sediment was resuspended in cold PBS or bovine serum to yield 700 to 2,000 microfilariae per 0.03 m*l*. Just prior to the feeding, ATP was added in the final concentration of 10^{-3} M.

Controls were the direct feeding of *Aedes aegypti* (Liverpool strain) on an infected dog and the artificial feeding of the mosquitoes with the microfilariae obtained from the venous blood of the same dog. The microfilaria count of the dog was 495 per 0.03 m*l* of peripheral blood. The mosquitoes were allowed to feed for 15 min on the infected dog under ketamine hydrochloride (Ketalar[R]) anesthesia. Venous blood was obtained from the vein of the hind limb of the dog with plastic syringes which were prepared by wetting the walls with 1,000 U/m*l* heparin. To each 5 m*l* of venous blood, 45 m*l* of cold one per cent saponin solution was added to attain hemolysis. The mixture was centrifuged for 5 min at 6,000 rpm, 0 C. The sediment was resuspended in cold saline and centrifuged under the same conditions twice. Then the sediment was resuspended in cold bovine serum and PBS to make 100 and 600 microfilariae per 0.03 m*l* respectively, to which 10^{-3} M ATP was added just prior to the feeding.

For feeding, 3 m*l* of solution was placed in the apparatus to a cage of 89 to 140 female mosquitoes. The mosquitoes were allowed to feed for 15 min at the ambient temperature of 25 ± 1 C. To count the number of microfilariae ingested, the mosquitoes were anesthetized and dissected under an operating microscope immediately after feeding. To observe the exsheathing of microfilariae, 5 to 10 mosquitoes of

each cage were anesthetized and dissected every hour from one to 8 hours after feeding except 7 hours. The content of the stomach was smeared on a glass slide (Figure 3), dried rapidly, fixed with methylalcohol and stained with Giemsa solution. Also 5 to 10 mosquitoes per cage were dissected every day from one to 13 days after feeding to observe the development of larvae. The larvae were classified into three developing groups according to the morphological characteristics described by Schacher (1962 a). Measurement of body length was done on the infective larvae recovered from the mosquitoes dissected 11 days after feeding.

Results

I. Response of *Aedes aegypti* to nucleotide solutions

Physiological saline without nucleotide was not detected in the stomach nor in the diverticula of *Aedes aegypti* (Table 1). Saline containing 10^{-4} M AMP was

Table 1 Response of *Aedes aegypti* to nucleotides in saline with localization and degree of intake of solutions†

Nucleotide	No. of mosquitoes			
	dissected	fed	in stomach (degree of intake)††	in diverticula (degree of intake)††
None	30	0	0	0
AMP 10^{-4}M*	29	5	5 (p, 3: s, 2)	1 (s, 1)
ADP 10^{-4}M*	29	14	14 (p, 10: s, 4)	0
ATP 10^{-4}M*	30	18	18 (f, 4: p, 13: s, 1)	0
AMP 10^{-3}M**	94	81	80 (f, 27: p, 53)	1 (s, 1)
ADP 10^{-3}M**	83	80	80 (f, 27: p, 53)	0
ATP 10^{-3}M**	86	73	73 (f, 20: p, 53)	0

† Artificial feeding at ambient temperature of 25–25.5 C.
†† f; fully distended: p; partially distended: s; slightly distended.
* A drop of 1% patent blue solution was added to each 2 m*l*.
** A drop of Indian ink was added to each 3 m*l*.

found in 5 of 29 mosquitoes, in 3 of which the stomach was partially distended, in two slightly, and in one the diverticula also contained small quantity of the solution. Saline containing 10^{-4} M ADP was found in 14 of 29 mosquitoes; in 10 of them the stomach was partially distended and in four slightly. The phagostimulant effect of 10^{-4} M ADP was more marked than that of 10^{-4} M AMP: The difference in the stomach engorgement of the solution was significant ($p<0.0254$) in the chi square test. Saline containing 10^{-4} M ATP was found in 18 of 30 mosquitoes; in four of them the stomach was fully distended, in 13 partially and in one slightly. ATP was more effective than AMP: The difference in the stomach engorgement between 10^{-4} M AMP and 10^{-4} M ATP was statistically significant ($p<0.0019$). When 10^{-3} M nucleotides were added to saline, more mosquitoes gorged and the

rates of full stomach distension were higher than those in 10^{-4} M concentrations (Table 1): Using the chi square test the differences in the stomach engorgement were significant in AMP ($p<0.0001$), ADP ($p<0.0001$) and ATP ($p<0.0096$).

PBS without nucleotides was detected in 4 of 30 mosquitoes, in one of which the stomach was fully distended, in one partially and in two slightly (Table 2). When 10^{-4} M AMP or 10^{-4} M ADP was added to PBS, the rate of stomach intake seemed to be a little higher than the solution without nucleotides, but the computed values of chi square were not statistically significant. PBS containing 10^{-4} M ATP was engorged by 23 of 29 mosquitoes, in 22 of which the stomach was fully distended. As a phagostimulant, ATP was significantly more effective than AMP ($p<0.0015$) or ADP ($p<0.0003$). On the dissection, sucrose solution was found to remain in the diverticula of the mosquitoes offered 10^{-3} M nucleotides in PBS. It might probably have inhibited the feeding of nucleotide solutions to a certain extent. Only 46 of 90 mosquitoes took 10^{-3} M AMP solution (Table 2). Although the rate of engorgement was significantly higher than the control solution ($p<0.0015$), the difference between 10^{-4} M AMP and 10^{-3} M AMP was not statistically significant. On the other hand, the difference between 10^{-4} M ADP and 10^{-3} M ADP was highly significant ($p<0.0002$). The rate of engorgement of 10^{-3} M ADP in PBS was higher than that of 10^{-3} M AMP in PBS: The difference was statistically significant ($p<0.032$). As a phagostimulant in PBS, 10^{-3} M ATP was more effective than 10^{-3} M ADP: The difference was also significant ($p<0.020$).

Table 2 Response to nucleotides in PBS with localization and degree of intake of solutions†

Nucleotide	No. of mosquitoes			
	dissected	fed	in stomach (degree of intake)††	in diverticula (degree of intake)††
None	30	4	4 (f, 1: p, 1: s, 2)	0
AMP 10^{-4}M*	30	9	9 (f, 1: p, 1: s, 7)	0
ADP 10^{-4}M*	30	7	7 (f, 7)	3 (s, 3)
ATP 10^{-4}M*	29	23	23 (f, 22: p, 1)	3 (p, 3)
AMP 10^{-3}M**	90***	46	46 (f, 19: p, 27)	0
ADP 10^{-3}M**	93***	63	63 (f, 34: p, 29)	0
ATP 10^{-3}M**	96***	80	79 (f, 34: p, 45)	1 (p, 1)

† Artificial feeding at ambient temperature of 22–24 C.

†† f; fully distended: p; partially distended: s; slightly distended.

* A drop of 1% patent blue solution was added to each 2 m*l*.

** A drop of Indian ink was added to each 3 m*l*.

*** Sucrose solution remained in diverticula.

Bovine serum was light red in color due to hemolysis. At the ambient temperature of 17 C, bovine serum without nucleotides was taken by 19 of 30 mosquitoes, in 18 of which serum was detected in the stomach and in three in the diverticula (Table 3). Only 8 of 30 mosquitoes took dog serum without nucleotides, which had been obtained without apparent hemolysis. The difference in the stomach engorgement

between bovine serum and dog serum was statistically significant ($p<0.0191$). The addition of 10^{-4} M AMP or 10^{-4} M ADP to bovine serum did not enhance the rate of feeding: The computed values of chi square were not statistically significant. On the other hand, the addition of 10^{-4} M ATP stimulated a little the stomach engorgement: The difference was statistically significant ($p<0.0487$). At the ambient temperature of 25 C, 39 of 100 mosquitoes gorged bovine serum without nucleotides; only in eight of them the stomach was fully distended and in 31 partially (Table 4). The addition of 10^{-3} M nucleotides to serum enhanced the rates of feeding and full stomach distension. The differences in the stomach engorgement were highly significant ($p<0.0001$) in the three nucleotides. However, the addition of 10^{-3} M AMP to bovine serum was not so effective as that of 10^{-3} M ADP or of 10^{-3} M ATP. When ADP or ATP was added, the rate of feeding was equal to that in rabbit defibrinated blood. The difference between 10^{-3} M AMP and rabbit defibrinated blood was highly significant ($p<0.0012$), while the computed values of

Table 3 Response to 10^{-4}M nucleotides in bovine serum† and to dog serum††

Nucleotide	No. of mosquitoes			
	dissected	fed	in stomach (degree of intake)**	in diverticula (degree of intake)**
None, bovine serum*	30	19	18 (f, 4: p, 2: s, 12)	3 (s, 3)
AMP 10^{-4}M*	29	18	18 (f, 4: p, 5: s, 9)	3 (p, 1: s, 2)
ADP 10^{-4}M*	28	22	22 (f, 6: p, 4: s, 12)	3 (p, 1: s, 2)
ATP 10^{-4}M*	29	25	25 (f, 20: s, 5)	2 (s, 2)
None, dog serum	30	8	8 (f, 1: p, 1: s, 6)	0

† Bovine serum was a little hemolysed.
†† Artificial feeding at ambient temperature of 17 C.
* A drop of 1% patent blue solution was added to each 2 m*l*.
** f; fully distended: p; partially distended: s; slightly distended.

Table 4 Response to 10^{-3}M nucleotides in bovine serum† and to rabbit defibrinated blood††

Nucleotide	No. of mosquitoes			
	dissected	fed	in stomach (degree of intake)**	in diverticula
None, bovine serum*	100	39	39 (f, 8: p, 31)	0
AMP 10^{-3}M*	95	74	74 (f, 40: p, 34)	0
ADP 10^{-3}M*	94	90	90 (f, 77: p, 13)	0
ATP 10^{-3}M*	100	96	96 (f, 75: p, 21)	0
None, rabbit defibrinated blood*	89	85	85 (f, 68: p, 17)	0

† Bovine serum was a little hemolysed.
†† Artificial feeding at ambient temperature of 25 C.
* A drop of Indian ink was added to each 3 m*l*.
** f; fully distended: p; partially distended: s; slightly distended,

chi square were not statistically significant in ADP and ATP. The rate of full stomach distension was also lower in AMP than in ADP, ATP or defibrinated blood (Table 4).

These results indicated that ATP was the most effective phagostimulant to *Aedes aegypti*, that ADP was next to ATP and that AMP was the least of three nucleotides. It was also clear that 10^{-3} M concentration was generally more effective than 10^{-4} M concentration.

II. Artificial feeding and development of larvae

The mosquitoes which fed directly on the dog with microfilaremia of 495 per 0.03 m*l* of peripheral blood became infected at a rate of 88.5 per cent (Table 5). At the dissection immediately after feeding, the average number of microfilariae in the stomach of the infected mosquito was 18.0 and the range was two to 75. The mosquitoes fed on PBS containing the microfilariae from the dog venous blood at the density of 600 per 0.03 m*l* became infected at a rate of 92.3 per cent. The number of microfilariae in the stomach of the infected mosquito was 21.5 on an average, varying from four to 47. The mosquitoes taken PBS containing the microfilariae from the jird peritoneal cavity at the density of 700 per 0.03 m*l* became infected at a rate of 33.3 per cent. The average number of microfilariae per the infected was 38.0 and the range was five to 204. When the density was raised to 1,000 in PBS, the infection rate was 77.7 per cent, and the average number was 204.0 with the range of four to 653. The microfilarial density of 1,500 in PBS produced the infection rate of 84.2 per cent and the average number of 336.6 with the range of one to 1,213. Thus, the infection rate seemed to be lower in the artificial feeding with PBS than in the direct feeding, if the microfilarial counts were similar in both groups.

Table 5 Infection rates in mosquitoes and numbers of microfilariae in stomach on dissection immediately after feeding

Source of mf.	Feeding medium*	Mf. count per 0.03 m*l*	No. of mosquitoes		Mean no. of mf. per infected (range)
			dissected	infected (%)	
Dog peripheral blood	(direct)	495	26	23 (88.5)	18.0 (2–75)
Dog venous blood	PBS	600	13	12 (92.3)	21.5 (4–47)
	bovine serum	100	12	5 (41.7)	5.2 (2–9)
Jird peritoneal cavity	PBS	700	27	9 (33.3)	38.0 (5–204)
		1,000	27	21 (77.7)	204.0 (4–653)
		1,500	19	16 (84.2)	336.6 (1–1,213)
	bovine serum	1,000	20	15 (75.0)	19.0 (1–79)
		1,500	20	20 (100)	180.2 (3–505)
		2,000	20	20 (100)	137.8 (1–1,002)

* As phagostimulant 10^{-3}M ATP was contained.

The average number of microfilariae per infected mosquito was greater and the individual count per mosquito varied more widely in the artificial feeding with PBS than in the direct feeding. The infection rates with bovine serum were similar to those with PBS (Table 5). The computed value of chi square was not statistically significant at the density of 1,000. In bovine serum, however, the average number of microfilariae per the infected seemed to be less and the individual count per mosquito varied less widely than in PBS.

The microfilariae from the dog venous blood exsheathed in the stomach of *Aedes aegypti* after the artificial feeding. Exsheathing reached the high percentage of 38.9 at one hour and that of 54.8 at 2 hours after the feeding with bovine serum (Table 6). Thereafter, the percentage of the exsheathed microfilariae among the ones remaining in the stomach ranged from 26.8 to 58.4. Exsheathing of the microfilariae from the dog venous blood occurred at lower rates with PBS than with bovine serum: The percentages were only 6.4 to 18.4 with PBS.

Table 6 Exsheathing of microfilariae in stomach after artificial feeding

Source of mf.	Feeding medium*	No. of dissected mosquitoes	No. of exsheathed mf./total no. of mf. (%) on hourly dissection after feeding**							
			1	2	3	4	5	6	8	total
Dog venous blood	PBS	50	39/610 (6.4)	19/156 (12.2)	51/440 (11.6)	33/399 (8.3)	20/140 (14.3)	N.D.	16/87 (18.4)	178/1,832 (9.7)
	bovine serum	52	21/54 (38.9)	51/93 (54.8)	15/56 (26.8)	34/81 (42.0)	31/70 (44.3)	66/113 (58.4)	21/53 (39.6)	239/520 (46.0)
Jird peritoneal cavity	PBS	31	2/740 (0.3)	0/111 (0)	5/257 (1.9)	16/433 (3.7)	16/693 (2.3)	9/312 (2.9)	1/988 (0.1)	49/3,534 (1.4)
	bovine serum	57	0/452 (0)	4/427 (0.9)	3/109 (2.8)	14/112 (12.5)	12/130 (9.2)	35/137 (25.5)	32/174 (18.6)	100/1,541 (6.5)

* As phagostimulant 10^{-3}M ATP was contained.

** Dissection was not done 7 hours after feeding.

The microfilariae from the jird peritoneal cavity exsheathed also in the stomach of mosquito, although less than one per cent of the microfilariae exsheathed at one hour and 2 hours after the feeding both with PBS and bovine serum. After 4 hours of feeding the percentage of the exsheathed microfilariae varied from 9.2 to 25.5 with bovine serum, while less than 4 per cent of microfilariae exsheathed with PBS. Thus, the microfilariae from the jird peritoneal cavity exsheathed at much lower rates than those from the dog venous blood with both media. The differences in the overall exsheathing between the microfilariae from the jird peritoneal cavity and those from the dog venous blood were highly significant ($p<0.0001$) in both media.

As shown in Table 7, the death rates of mosquitoes were lower in the groups taken the microfilariae from the jird peritoneal cavity than in the groups received the microfilariae from the dog venous blood, although the density of microfilariae in the feeding medium and the average number of microfilariae taken up by a mosquito were higher in the former groups. The difference in the death rate between the

Table 7 Numbers of deaths in mosquitoes 1 to 11 days after artificial feeding

Source of mf.	Feeding medium*	Mf. count per 0.03 ml	Total no. of mosquitoes	Mean no. of mf. per infected (range)**	No. of mosquitoes died on days after feeding											
					1	2	3	4	5	6	7	8	9	10	11	total
Dog venous blood	PBS	600	89	21.5 (4–47)	1	3	24	7	1	0	2	0	0	0	0	38 (42.7)
	bovine serum	100	114	5.2 (2–9)	0	8	8	22	3	0	4	0	0	0	0	45 (39.5)
Jird peri-toneal cavity	PBS	700	132	38.0 (5–204)	0	0	1	3	7	6	8	3	5	1	0	34 (25.8)
		1,000	122	204.0 (4–653)	0	3	4	0	2	2	0	0	0	0	0	11 (9.0)
		1,500	105	336.6 (1–1,213)	1	9	3	0	0	0	1	2	1	0	0	17 (16.2)
	bovine serum	1,000	140	19.0 (1–79)	1	0	1	0	5	5	4	10	3	1	1	31 (22.1)
		1,500	97	180.2 (3–505)	4	1	1	0	0	0	0	1	1	1	0	9 (9.3)
		2,000	100	137.8 (1–1,002)	5	2	0	0	0	0	0	0	0	1	0	8 (8.0)

* As phagostimulant 10^{-3}M ATP was contained.

** Numbers immediately after feeding.

group received the microfilariae from the dog at the density of 600 per 0.03 ml in PBS and the group taken those from the jird at the density of 700 in PBS was statistically significant ($p<0.013$). Also the difference was significant ($p<0.0044$) between the group received the microfilariae from the dog at the density of 100 in bovine serum and that taken the microfilariae from the jird at the density of 1,000 in bovine serum. The mosquitoes received the microfilariae from the dog most frequently died 3 or 4 days after feeding. In the groups taken those from the jird at the density of 700 in PBS and at that of 1,000 in bovine serum, most deaths occurred 5 to 7 days after feeding. When the density was higher, most deaths occurred much earlier, within one to 3 days of infection.

On and after one day of infection, the infection rate of mosquitoes taken the microfilariae from the dog venous blood at the density of 600 in PBS was as high as that in the group which fed directly on the dog (Table 8). In the group offered the microfilariae from the dog at the low density of 100 in bovine serum, the infection rate was as low as 12.9 per cent 10 to 13 days after feeding. In the groups given the microfilariae from the jird in PBS, the infection rates were low; although the density of those from the jird was raised to 1,000 or 1,500, the rates at the period between 10 to 13 days of infection seemed to be lower than that of the microfilariae from the dog offered at the density of 600. Also the rates were low in the groups received the microfilariae from the jird in bovine serum. The infection rate was 30 per cent in the mosquitoes given the microfilariae from the jird at the density of 1,000 in bovine serum 10 to 13 days after feeding, seemingly higher than that in the mosquitoes taken the microfilariae at the density of 100. But the difference was not statistically significant. When the density of those from the jird was raised to 1,500 or 2,000 in bovine serum, the rate at the same period became significantly higher ($p<0.039$ at 1,500; $p<0.0017$ at 2,000) than that of the microfilariae from the dog given at the density of 100.

Table 8 Infection rates in mosquitoes one to 13 days after direct or artificial feeding

Source of mf.	Feeding medium*	Mf. count per 0.03 ml	No. of mosquitoes Infected/dissected (%) for periods		
			1–5 days	6–9 days	10–13 days
Dog peripheral blood	(direct)	495	18/25 (72.0)	16/20 (80.0)	9/10 (90.0)
Dog venous blood	PBS	600	13/15 (86.7)	12/13 (92.3)	9/10 (90.0)
	bovine serum	100	5/14 (35.7)	2/12 (16.7)	4/31 (12.9)
Jird peritoneal cavity	PBS	700	25/61 (41.0)	10/42 (23.8)	25/76 (32.9)
		1,000	42/63 (66.7)	29/42 (69.0)	51/75 (68.0)
		1,500	20/27 (74.1)	17/22 (77.3)	11/20 (55.0)
	bovine serum	1,000	14/28 (50.0)	2/21 (9.5)	12/40 (30.0)
		1,500	22/27 (81.5)	11/21 (52.4)	10/20 (50.0)
		2,000	21/27 (77.8)	13/20 (65.0)	14/25 (56.0)

* As phagostimulant 10^{-3}M ATP was contained.

The individual counts of larvae recovered from the mosquitoes fed directly on the dog did not vary widely throughout the development of larvae (Table 9). In this group, the average number of larvae per mosquito was 5.7 to 14.0 during the period between one and 3 days of infection and did not fluctuate widely in the subsequent period of infection. In the mosquitoes given the microfilariae from the dog venous blood at the density of 600 per 0.03 ml in PBS, the average number of larvae per mosquito was equal to or somewhat higher than that of direct feeding group, although the individual counts of larvae varied more widely. In the mosquitoes fed on the microfilariae from the dog venous blood at the low density of 100 in bovine serum, only a few larvae were recovered even in the early period of infection.

The average number of larvae per mosquito was much lower in the group offered the microfilariae from the jird peritoneal cavity at the density of 700 per 0.03 ml in PBS than in the group taken those from the dog venous blood at the density of 600 (Table 9): Only 2 of 179 mosquitoes dissected during 13 days of infection harbored 10 larvae or more. When the microfilarial density in PBS was raised to 1,000, the average number approached to that of the direct feeding group. In the group fed on PBS containing the microfilariae at the density of 1,500, the average number was higher than that of the direct feeding group during the period between one and 6 days of infection and equal to that of the latter subsequently. In the groups given the microfilariae from the jird peritoneal cavity with bovine serum, the average number of larvae per mosquito was far below that of the direct feeding group. In the artificial feeding groups, especially those given the microfilariae with PBS, the individual counts of larvae recovered from the mosquitoes varied widely, more frequently in the early period of infection than in the late period.

Table 9 Numbers of larvae developed in mosquitoes after direct or artificial feeding

Source of mf.	Feeding medium*	Mf. count per 0.03 m*l*	Mean no. of larvae per infected mosquito (range) on days after feeding					
			1	2	3	4	5	6
Dog peripheral blood	(direct)	495	14.0 (4–26)	5.7 (1–13)	9.3 (2–16)	4.7 (1–8)	8.3 (4–12)	3.0 (1–6)
Dog venous blood	PBS	600	9.3 (8–12)	19.3 (3–35)	27.5 (4–51)	5.0 (2–7)	9.5 (3–16)	30.0 (2–57)
	bovine serum	100	4.0 (4–4)	1.0 (1–1)	6.0 (6–6)	4.0 (4–4)	1.0 (1–1)	ND
Jird peritoneal cavity	PBS	700	3.0 (1–4)	4.0 (1–8)	1.3 (1–2)	1.6 (1–3)	1.0 (1–1)	1.0 (1–1)
		1,000	5.0 (1–9)	18.6 (1–72)	18.0 (2–48)	9.8 (2–25)	5.4 (1–19)	10.7 (1–23)
		1,500	50.5 (16–115)	4.0 (2–8)	21.3 (1–61)	15.0 (3–30)	16.3 (13–20)	20.3 (3–57)
	bovine serum	1,000	2.3 (1–4)	1.0 (1–1)	2.0 (1–3)	2.5 (2–3)	2.0 (1–3)	ND
		1,500	27.0 (3–61)	4.3 (3–7)	4.5 (1–12)	6.3 (1–16)	4.0 (3–6)	5.0 (1–11)
		2,000	3.8 (2–8)	6.3 (2–12)	3.8 (1–7)	3.8 (1–8)	5.3 (1–13)	2.5 (1–4)

Source of mf.	Mean no. of larvae per infected mosquito (range) on days after feeding						
	7	8	9	10	11	12	13
Dog peripheral blood	8.3 (7–9)	4.8 (2–10)	6.8 (4–12)	9.6 (3–16)	10.8 (5–20)	ND	ND
Dog venous blood	17.3 (2–41)	10.0 (4–16)	6.7 (2–12)	14.7 (3–21)	11.8 (4–21)	ND	ND
	1.0 (1–1)	1.0 (1–1)	ND	1.0 (1–1)	1.0 (1–1)	ND	ND
Jird peritoneal cavity	6.0 (3–9)	2.7 (1–5)	8.5 (2–15)	1.0 (1–1)	1.1 (1–2)	1.8 (1–3)	2.7 (1–10)
	1.9 (1–3)	8.2 (1–17)	8.7 (1–21)	9.2 (1–25)	10.8 (1–44)	5.8 (1–18)	1.8 (1–4)
	8.3 (1–24)	15.0 (3–45)	6.4 (1–14)	6.3 (3–15)	10.6 (5–15)	ND	ND
	ND	1.0 (1–1)	ND	1.0 (1–1)	1.6 (1–3)	ND	ND
	3.0 (1–7)	2.6 (2–3)	1.5 (1–2)	3.7 (2–5)	4.6 (2–10)	ND	ND
	6.0 (4–8)	4.5 (1–7)	3.0 (1–6)	2.2 (1–4)	7.0 (1–16)	ND	ND

* As phagostimulant 10^{-3}M ATP was contained.

As shown in Figure 4, stage I larvae were detected in the thoracic muscle of the mosquitoes fed directly on the dog and dissected one to 5 days after feeding. Only stage II larvae were found in the direct feeding mosquitoes dissected 6 to 9 days after feeding. In the mosquitoes dissected on 10 days of infection, 72.9 per cent of larvae were still in stage II and localized in the thoracic muscle, while 8.9 per cent of larvae had reached stage III and were detected in the head, and 18.8 per cent had also reached stage III and were found in the abdomen. The mosquitoes dissected on 11 days harbored stage II larvae in the thoracic muscle (11.6%), and stage III larvae in the head (74.4%), in the thorax (4.7%) and in the abdomen (9.3%). The larvae in the mosquitoes received the microfilariae from the dog venous blood followed the gradual development of those in the direct feeding mosquitoes without much delay. Only stage I larvae were found in the thoracic muscle of the mosquitoes which took up the microfilariae from the dog venous blood in PBS and were dissected one to 5 days after feeding. In this group of mosquitoes dissected on 6 days of infection, 3.3 per cent of larvae were still in stage I and 96.7 per cent had reached stage II in the thoracic muscle. All larvae found in the mosquitoes dissected 7 to 9 days after feeding were in stage II and localized in the thoracic muscle. On 10 days of infection 47.7 per cent of larvae were still in stage II localized in the thoracic muscle, while 18.2 per cent had attained stage III in the head, 29.5 per cent had attained the same stage in the thorax and 4.6 per cent in the abdomen. The mosquitoes dissected on 11 days had stage III larvae in the head (69%), thorax (11.3%) and abdomen

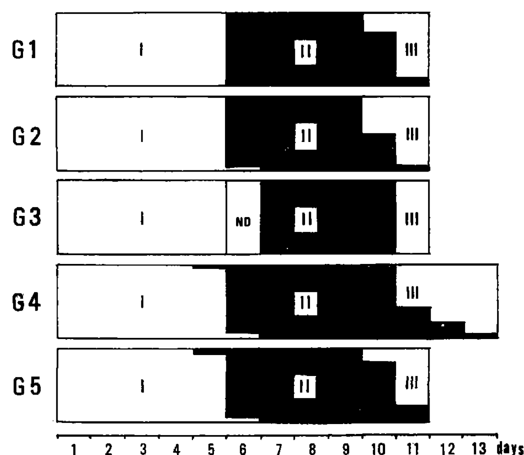

Figure 4 Development of *Brugia pahangi* larvae in *Aedes aegypti* after direct and artificial feeding.

G 1, direct feeding on an infected dog; G 2, microfilariae from dog venous blood fed artificially with PBS; G 3, microfilariae from dog venous blood fed artificially with bovine serum; G 4, microfilariae from jird peritoneal cavity fed artificially with PBS (three experiments with different microfilarial densities combined); G 5, microfilariae from jird peritoneal cavity fed artificially with bovine serum (three experiments with different microfilarial densities combined); I, stage I larvae; II, stage II larvae; III, stage III larvae.

(2.7%), besides stage II larvae in the thoracic muscle (7%). The number of infected mosquitoes were small, only 11, in the group which were offered the microfilariae from the dog venous blood in bovine serum and dissected one to 11 days after feeding. In this group all larvae found at one to 5 days' dissection were in stage I. Only stage II larvae were detected 7 to 10 days after feeding. All larvae reached stage III on 11 days.

In the group of mosquitoes received the microfilariae from the jird peritoneal cavity in PBS at the density of 700, only stage I larvae were found on 5 days of infection, and only stage II larvae were detected on 6 days. On the other hand, stage II larvae were found on 5 days at a rate of 3.7 per cent in the group offered the microfilariae from the jird in PBS at the density of 1,000 and at a rate of 2.5 per cent in the group offered at 1,500. On 6 days of infection, 10.3 per cent of larvae were still in stage I in the group taken the microfilariae at 1,000 in PBS, and all larvae reached stage II in the group taken at 1,500. Stage II larvae were found at a rate of 12.5 per cent in the group received the microfilariae from the jird in bovine serum at the density of 1,500 on 5 days, while only stage I larvae were detected in the group received at 1,000 and that at 2,000. On 6 days 6.7 per cent were still in stage I in the group received the microfilariae at 1,500, and all larvae reached stage II in that at 2,000. Thus, some of the microfilariae from the jird peritoneal cavity reached stage II one day earlier than the microfilariae from the dog. On the other hand, stage I larvae were still found on 6 days in the groups taken the microfilariae from the jird peritoneal cavity as in the group received the microfilariae from the dog venous blood. The development of the microfilariae from the jird to stage II seemed to be not influenced by the density of microfilariae in the medium, nor by the nature of the medium (i.e. PBS or bovine serum).

In the groups received the microfilariae from the jird in PBS at the density of 700 and 1,000, all larvae were still in stage II on 10 days of infection, and stage II larvae were 25.0 per cent and 43.4 per cent respectively on 11 days. Stage II larvae were still found on 12 days at a rate of 11.1 per cent and 20.0 per cent respectively. In the former group 7.4 per cent of larvae remained at stage II even on 13 days of infection. In the group taken the microfilariae from the jird in PBS at the density of 1,500, stage II larvae were 96.0 per cent on 10 days and 17.6 per cent on 11 days. In the mosquitoes received the microfilariae from the jird in bovine serum, the rate of stage II larvae on 10 days was 33.3 per cent at the density of 1,000, 81.8 per cent at 1,500 and 72.2 per cent at 2,000, and that on 11 days was 28.6 per cent, 25.0 per cent and 17.0 per cent respectively. Thus, the development of the microfilariae from the jird peritoneal cavity into the final form in the mosquito seemed to be slower than that of the microfilariae in the direct feeding on the dog and that of the microfilariae from the dog venous blood (Figure 4). Also the rate of stage III larvae situated in the head was 26.7 to 41.9 per cent on 11 days in the groups taken the microfilariae from the jird, much lower than that in the groups fed on the microfilariae from the dog.

On the dissection 10 to 11 days after feeding, 9 of 10 mosquitoes which fed directly on the dog harbored the infective (i.e. stage III) larvae (Table 10). The number of infective larvae per infected mosquito ranged from one to 17 with an

Table 10 Numbers of stage III larvae in mosquitoes 10 to 11 days after feeding and lengths of larvae

Source of mf.	Feeding medium*	Mf. count per 0.03 ml	No. of mosquitoes		Mean no. of stage III larvae per infected (range)	Range of lengths (mean)**
			dis-sected	with stage III larvae (%)		
Dog peripheral blood	(direct)	495	10	9 (90.0)	5.7 (1–17)	1,422–1,676 (1,464.3)
Dog venous blood	PBS	600	10	9 (90.0)	9.9 (1–21)	1,294–1,627 (1,431.6)
	bovine serum	100	31	3 (9.7)	1.0 (1–1)	ND
Jird peritoneal cavity	PBS	700	76	18 (23.7)	2.2 (1–9)	1,451–1,789 (1,584.2)
		1,000	75	43 (57.3)	5.8 (1–21)	
		1,500	20	8 (40.0)	7.8 (1–11)	
	bovine serum	1,000	40	10 (25.0)	1.2 (1–2)	1,235–1,643 (1,440.0)
		1,500	20	8 (40.0)	3.3 (1–7)	
		2,000	25	11 (44.0)	5.0 (1–13)	

* As phagostimulant 10^{-3}M ATP was contained.

** Lengths in micron on 11 days.

average of 5.7. The length of infective larva on 11 days varied between 1,422 μ and 1,676 μ with an average of 1,464.3 μ. In the group taken the microfilariae from the dog venous blood in PBS at the density of 600, the percentage of mosquitoes harboring the infective larvae was 90 per cent, and the average number of infective larvae was 9.9, somewhat higher than the direct feeding group. In the mosquitoes taken the microfilariae at the density of 100, the infection rate was low and the number of larvae was small. In the groups received the microfilariae from the jird, the percentage of mosquitoes harboring the infective larvae was 23.7 to 57.3 per cent, lower than that in the mosquitoes fed directly on the dog and in those offered the microfilariae from the dog in PBS. The average number of infective larvae was also smaller in the groups received the microfilariae from the jird than in the group taken the microfilariae from the dog at the density of 600. The lengths of infective larvae in the groups received the microfilariae from the jird in bovine serum were similar to those in the group fed on the microfilariae from the dog in PBS. The length of infective larva in the mosquitoes offered the microfilariae from the jird in PBS varied between 1,451 μ and 1,789 μ with an average of 1,584.2 μ

Discussion

A new apparatus for feeding mosquitoes is small and easy of handling without a sophisticated self-contained heater and thermistor. Its construction is economical and involves no special techniques, using a plain laboratory glassware, mouse skin

etc. It can be used with any mosquito cage that has a top hole. The capacity for feeding medium is approximately 10 m*l*. When the conservation of medium is important, 2 m*l* or less can be used. Thus, the usefulness of the new apparatus is demonstrated in the present studies.

In the present studies ATP is the most effective phagostimulant of the nucleotides dissolved in saline, PBS or bovine serum. Hosoi (1959) reported that AMP and ADP were more effective than ATP to *Culex pipiens pallens* with a kind of forcible feeding. The difference in the species of mosquitoes and/or feeding techniques may have caused disagreement between the results. The fact that nucleotides in 10^{-3} M concentration are generally more effective than those in 10^{-4} M concentration is in accord with observations by Rutledge *et al.* (1964) that 0.005 M was near the optimum of ATP to Bangkok strain of *Aedes aegypti*. Thus, 10^{-3} M ATP is an adequate phagostimulant for the artificial feeding of Liverpool strain of *Aedes aegypti*.

Some microfilariae from the dog venous blood become inactive within 30 min in 5 per cent glucose solution or saline both containing 10^{-3} M nucleotides at 37 C, while inactive microfilariae are detected after 90 min in PBS containing 10^{-3} M nucleotides and after 20 hours in bovine serum containing them (unpublished data). So, PBS and bovine serum are suitable for the media for microfilarial feeding.

It is suggested that microfilariae are maldistributed in PBS, twining round debris, by the findings that the infection rates in mosquitoes are lower for the artificial feeding with PBS than for the direct feeding and that the individual count of microfilariae per mosquito varies widely in that with PBS, when the microfilarial densities are similar in both groups. In the artificial feeding with bovine serum, the infection rates are low as in that with PBS, although the microfilarial densities are higher than in the direct feeding group. However, the average numbers of microfilariae per infected mosquito are lower and the individual count per mosquito varies less widely with bovine serum than with PBS. It seems likely that the maldistribution of microfilariae is less in bovine serum than in PBS, probably due to the viscosity of serum.

The exsheathing of microfilariae from both the dog venous blood and the jird peritoneal cavity occurs higher in the artificial feeding with bovine serum than in that with PBS. The exsheathing may be disturbed in the mosquitoes taken up a number of microfilariae by the overcrowding in the stomach: it is more frequent with PBS than with bovine serum as indicated by the individual count per mosquito. It is also possible that the viscosity of serum facilitates the exsheathing in the stomach. Aoki (1971 a, b) and Aoki *et al.* (1976) reported that microfilariae exsheathed well on agar pads. The findings support the view that the exsheathing is switched on by the change of milieu toward solidification.

The death rates of mosquitoes are lower in the groups taken the microfilariae from the jird peritoneal cavity than in the groups received those from the dog venous blood. It is of interest to speculate whether the developing larvae inflict damage on mosquitoes (Rosen, 1955; Wharton, 1957; Townson, 1970; Hockmeyer *et al.*, 1975; Christensen, 1978), and whether some microfilariae from the jird are inadequate to the development, which results in the low death rate of mosquitoes. It may be also that most deaths occurring one to 3 days after feeding in the group taken high density of microfilariae are brought by a number of microfilariae penetrating the stomach

wall and developing in the thoracic muscle as stage I larvae.

On and after one day of infection, the rates of infected mosquitoes are low in the group taken the microfilariae from the dog venous blood at the density of 100 per 0.03 *ml* in bovine serum: it seems likely that the low rates of infection are brought by the low density of microfilariae offered. In the artificial feeding groups, especially those with PBS, individual counts of larvae vary widely one to 3 days of infection. It probably reflects the fact that the number of microfilariae taken varies greatly among the mosquitoes in the same groups.

The microfilariae from the jird peritoneal cavity exsheathe in both PBS and bovine serum at lower rates than those from the dog venous blood. The rate of infected mosquito is low in the groups received the jird microfilariae: 10 to 13 days after feeding, the rate in the group offered the jird microfilariae at the density of 1,000 is not significantly different from that in the group taken the dog microfilariae at the density of 100. The average number of larvae per infected tends to be low in the group taken the jird microfilariae. These findings suggest that the microfilariae from the jird peritoneal cavity includes the ones inadequate to the development in the mosquitoes, such as the newborn and the aged, at a higher rate than those from the dog venous blood.

Some microfilariae from the jird peritoneal cavity reach stage II one day earlier than the microfilariae from the dog. However, the development of the microfilariae from the jird to stage III is generally slower than that of the microfilariae from the dog. It probably causes the low rate in the mosquitoes harboring the stage III larvae and the low average number of stage III larvae per infected in the group received the microfilariae from the jird and dissected 10 to 11 days after feeding. It is clear from the present findings that the microfilariae develop to the stage III larvae in the mosquitoes which have not taken blood, serum or protein meals, and that the infective larvae sometimes become even longer with PBS.

Brugia pahangi inoculated, localized and producing microfilariae intraperitoneally and the microfilariae thus produced and localized in the peritoneal cavity seem to follow an aberrant mode of development. The present studies, however, have revealed that the microfilariae from the jird peritoneal cavity can reach stage III in *Aedes aegypti*, although at a lower rate than the microfilariae produced by the lymphatic adults in a natural host.

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スナネズミ腹腔より得た *Brugia pahangi* ミクロフィラリアの *Aedes aegypti* における発育

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蚊に対する人工摂取装置を新たに考案した。製作に特殊な技術を要せず，使用に便利である。その装置を用いて，*Aedes aegypti* の胃内摂取に対するヌクレオチドの影響と，スナネズミ腹腔ならびにイヌ静脈血より得たミクロフィラリアの，*Aedes aegypti* における発育の比較を行った。摂食刺戟物としては，用いたヌクレオチドの中で，10^{-3} M ATP が最も有効である。イヌ静脈血より得たミクロフィラリアと同じく，スナネズミ腹腔より得たものは，第 III 期幼虫（感染幼虫）に成長することが証明されたが，その率はイヌ静脈血より得たものより低い。また，血液，血清，蛋白を食物として摂取していない蚊においても，ミクロフィラリアが感染幼虫になることが証明された。

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COMPARATIVE SENSITIVITY OF FOCUS COUNTING METHOD IN THE INFECTIVITY TITRATION OF DENGUE VIRUSES AND IN THE NEUTRALIZATION TEST[1]

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Abstract: Infectivity titrations were performed for each of the 4 prototypes of dengue (DEN) viruses by focus counting on BHK21 cells (FFU), by plaque formation on LLC–MK_2 cells (PFU), and by intracerebral inoculation into suckling mice (LD_{50}). The FFU titers were almost similar as LD_{50} for all the 4 types of DEN and these titers were also similar to PFU except type 2 DEN (DEN–2), which showed lower PFU than FFU or LD_{50}. Neutralization (N) tests were performed for standard anti-DEN sera by 50 per cent focus reduction (FR_{50}) and by 50 per cent plaque reduction (PR_{50}) methods. Both values were almost the same except anti-DEN–2, whcih showed higher PR_{50} than FR_{50}. The principle of these methods were also applied to Japanese encephalitis and chikungunya viruses. Hemagglutination-inhibition (HI) and FR_{50}–N tests on several human sera indicated that FR_{50} is more specific and sensitive than HI to detect type-specific antibodies.

INTRODUCTION

Infections due to dengue (DEN) viruses of 4 different serotypes are very important diseases in Southeast Asia, causing sometimes severe hemorrhagic fevers accompanied by shock and death of infected children (Hammon *et al.*, 1960; Halstead, 1966). Hemagglutination-inhibition (HI) test has most routinely been used in the serodiagnosis and seroepidemiology of DEN infections (Nimmanitya *et al.*, 1969; Halstead *et al.*, 1969; Fukunaga *et al.*, 1974b), because of its simplicity and rapidity (Hammon and Work, 1964). However, high cross-reactivity by HI test among members of flavivirus, or group B arbovirus (Casals, 1957; Clarke and Casals, 1965; Melnick, 1974) does not often tell anything but flavivirus infection in Southeast Asia, where multiple flavivirus infections coexist. Although neutralization (N) test is most specific among serological reactions (Hammon and Work, 1964), the test has not been used in a large scale, because of the difficulty in the infectivity titration of DEN viruses. The standard method by intracerebral inoculation into suckling mice to record LD_{50} requires a long observation period and a large space for animal experiment. Even the more recent application of cell culture techniques require fairly long incubation before the final results are obtained, which are, therefore, not

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always guaranteed. In this laboratory we have developed a rapid titration of DEN virus infectivity by counting foci of infected BHK21 cells stained by indirect immunofluorescent (FA) or peroxidase-anti-peroxidase (PAP) technique and applied the method to assay N antibody (Igarashi and Mantani, 1974; Okuno *et al.,* 1977, 1978). In this report we describe the relative sensitivity of focus counting method in the infectivity titration of DEN viruses compared with other standard methods, and also the relative sensitivity of focus reduction N test, and discuss about its application to human serum specimens.

Materials and Methods

Viruses: Following DEN virus strains were used: type 1 (DEN–1) Hawaiian, type 2 (DEN–2) New Guinea B, type 3 (DEN–3) H–87, and type 4 (DEN–4) H–241. The origins and passage histories of these viruses were already described as well as the method to prepare infected suckling mouse brain (SMB) stock viruses (Igarashi, 1978). The Nakayama strain of Japanese encephalitis (JE) virus was grown in adult mouse brains, and chikungunya (CHIK) virus, African strain, was plaque-purified and seed virus was prepared in BHK21 cells (Igarashi and Fukai, 1969).

Cells: BHK21 cells were grown at 37 C with 10 per cent calf serum in Eagle's (1959) minimal essential medium (MEM). LLC–MK_2 cells were obtained from Dr. S. Inoue, National Institute of Health, Tokyo, and were grown with 10 per cent fetal calf serum in MEM. These cells were transferred every 4 days at cell density of 10^5 cells/m*l* and was incubated at 37 C in 5 per cent CO_2 atmosphere. Monolayers were ready after 3–4 days in dishes and after 2 days in 8-chamber slides (Miles, Ill. USA).

Infectivity titrations: Focus counting method is a rapid infectivity titration on BHK21 cells prepared in 8-chamber slides by counting the number of groups of cells containing virus antigen which was revealed either by FA or PAP method (Igarashi and Mantani, 1974; Okuno *et al.,* 1977, 1978). In the case of CHIK virus, tragacanth gum (Wako Pure Chemicals Co. Osaka, Japan) was introduced to final 1 per cent in the medium in order to prevent spreading of the secondary foci. Specimens were harvested 16 hours for CHIK, 24 hours for JE, 40 hours for DEN–2, 48 hours for DEN–4, and 72 hours for DEN–1 and DEN–3 viruses, respectively, after incubation at 37 C. Titers were shown as focus forming units (FFU) per m*l*. Plaque formation on LLC–MK_2 cells was performed by the method of Russell *et al.* (1967) and titers were shown as plaque forming units (PFU) per m*l*. LD_{50} titration in SMB was performed by inoculation of serially 10-fold diluted specimens into the brains (0.01 m*l*/brain) and titers were expressed as LD_{50}/m*l*.

Human serum specimens and neutralization (N) test: Several individual human sera were obtained from persons who either had been living in Southeast Asia or had some chances to contact with DEN or CHIK viruses. All the sera were heat-inactivated (56 C, 30 min) before the test. Serially diluted test serum was mixed with the equal volume of virus specimen diluted to 200 FFU/0.1 m*l* for FR_{50}, or 200 PFU/0.2 m*l* for PR_{50} test. After incubation at 28 C for 2 hours, residual virus infectivity was assayed by FFU (0.1 m*l*/well), or by PFU (0.2 m*l*/dish) assay. Focus or plaque

reduction percent was plotted on a probit chart against the logarithm of the serum dilutions. The N titer of the test serum was shown either as 50 per cent focus reduction (FR_{50}), or 50 per cent plaque reduction (PR_{50}), the reciprocal of the serum dilution which will reduce the focus or plaque number to 50 per cent of the control without antiserum (Russell *et al.,* 1967; Okuno *et al.,* 1978).

Hemagglutination-inhibition (HI) test: The method of Clarke and Casals (1958) was followed with modification to microtiter system (Sever, 1962).

Antiserum: Anti-DEN sera were prepared in rabbits by 2 intramuscular inoculations with one month interval of partially purified virus with complete Freund's adjuvant (Okuno *et al.,* 1978).

Results

Standard prototype DEN viruses were assayed for their infectivity by FFU on BHK21 cells, by PFU on LLC-MK_2 cells and by LD_{50} in SMB, and the results are summarized in Table 1. The FFU titers were almost the same as LD_{50} for all the 4 types of DEN viruses, and these titers were also similar to PFU for DEN-1, DEN-3 and DEN-4 viruses. However, DEN-2 showed significantly lower PFU than FFU or LD_{50} titers. This difference was observed particularly with New Guinea B strain but it was less when TR1751 strain of DEN-2 virus was used.

N test of the standard anti-DEN rabbit sera were performed either by FR_{50} or PR_{50} method against the homologous DEN viruses, and the results are shown in Table 2. Both the FR_{50} and PR_{50} titers were almost the same for anti-DEN-1, anti-DEN-3 and anti-DEN-4 sera, however, PR_{50} titer of anti-DEN-2 serum was significantly higher than its FR_{50}. This may reflect the relative insensitivity of PFU assay on LLC-MK_2 for DEN-2 virus, but detailed mechanism is not known yet.

Table 1 Relative infectivity (log/m*l*) of different dengue viruses

Virus	BHK21 FFU	LLC-MK_2 PFU	SMB LD_{50}
DEN-1	6.2	6.6	6.8
DEN-2	8.3	7.1	8.3
DNE-3	5.7	5.8	6.7
DEN-4	6.3	6.2	6.4

Table 2 Neutralization titers of anti-dengue sera

Antiserum	FR_{50} on BHK21	PR_{50} on LLC-MK_2
Anti-DEN-1	940	620
Anti-DEN-2	820	2500
Anti-DEN-3	1,100	1,380
Anti-DEN-4	190	320

Limitted numbers of human sera from persons possibly exposed to DEN or CHIK virus were assayed for their antibody titers by HI test and the results are shown in Table 3. All the sera from residents of Southeast Asian countries had HI antibodies broadly reactive to all the 4 types of DEN as well as JE antigens. One Japanese who had previously been immunized with JE and yellow fever vaccine and who had suffered from an apparent DEN infection (No. 18) also showed the broadly reactive pattern. On the other hand, another Japanese who had received JE vaccine and who experienced an apparent DEN symptom showed monospecific reaction to

Table 3 HI titers of human sera

Serum No.	Permanent residence	Age Sex	Antigen					
			DEN-1	DEN-2	DEN-3	DEN-4	JE	CHIK
1	Thai	40M	20	40	20	80	20	20
2	Thai	39M	40	40	40	80	40	—*
3	Korea	34M	—	—	—	—	—	—
4	Japan	41M	—	—	—	—	—	—
5	Japan	32F	—	—	—	—	—	—
9	Thai	28M	40	80	80	80	40	—
13	Thai	40F	160	160	80	80	40	—
17	Burma	32F	160	320	80	20	20	—
18	Japan	36M	80	20	20	40	20	—
19	Japan	28M	80	—	—	—	—	—
23	Philippines	29F	1,280	320	160	80	80	—
24	Japan	55M	—	—	—	—	—	—
25	Japan	54M	—	—	—	—	—	—
26	Indonesia	30F	40	20	20	20	20	—

* less than 20.

DEN-1 antigen (No. 19). No other Japanese or Korean had any positive HI antibody against DEN or JE antigen.

These same sera were assayed for their N antibody titers by FR_{50} test and the results are shown in Table 4. Compared with the results in Table 3, two characteristic aspects could be pointed out. (i) The N test is more specific than HI, for example, serum No. 1 reacted broadly by HI, however the FR_{50} assay clearly indicated speficity to DEN-2 virus. Also, serum No. 18, though reacting broadly by HI, showed more specific reaction to DEN-1 virus by N test. (ii) The N test is, in general, more sensitive than HI to detect the presence of type-specific antibodies. There are 2 sera for DEN, 4 sera for JE and 6 sera for CHIK virus, which were negative by HI but positive by N test. Because of the broad reactivity of HI test and higher specificity of N test, several sera showed negative or lower N titer even when HI test showed positive or higher titers (Serum No. 13 for JE; No. 18 for DEN-2, 3 and 4; No. 23 for DEN-1). Serum No. 3 was obtained from a person working on DEN-4 virus in the laboratory without any obvious symptoms, and sera Nos. 4 and 5 were obtained from investigators dealing with CHIK virus. The data on these sera indicate some chances of laboratory infection by these viruses and also the importance of N test to detect such accidental infections because HI did not tell positive results for these cases. Serum No. 25 was obtained from a person who had been experimentally infected, more than 35 years ago, with dengue virus which was isolated during the dengue epidemic in Osaka in 1942 and was passaged several times in experimental animals (Taniguchi, 1943; Taniguchi *et al.*, 1951). Since he did not experience any overt DEN symptom afterward, the presence of anti-DEN-1 N antibody in the serum No. 25 indicates that the epidemic in Osaka, 1942, was pre-

Table 4 Neutralization titers (FR_{50}) of human sera

Serum No.	Previous history				Virus					
	Vaccine		Overt Dis*	Lab Exp**						
	JE	YF			DEN-1	DEN-2	DEN-3	DEN-4	JE	CHIK
1					18	960	16	64	45	44
2					270	150	420	350	84	88
3				DEN-4	—***	—	—	105	—	—
4	+	+		CHIK	—	33	—	—	4,000	4,000
5	+			CHIK	—	—	—	—	2,600	1,200
9					55	230	330	205	92	950
13					770	1,450	580	72	—	740
17					460	1,150	250	100	33	78
18	+	+	+		460	15	—	80	420	—
19	+		+		5,400	—	—	—	280	—
23	+				76	820	230	125	1,200	—
24	+				16	10	12	94	21	16
25	+		+		200	—	32	—	570	1,550
26	+				16	26	110	82	1,750	2,400

* Typical dengue fever.
** Any experience with laboratory experiments using viruses indicated.
*** less than 10.

sumably due to type 1 DEN virus. Long-lasting presence of anti-DEN N antibodies has been described (Halstead, 1974; Fujita, 1977).

Discussion

Considering the public health importance of dengue hemorrhagic fever in Southeast Asia (Chow *et al.,* 1966), it is necessary to establish a simple, rapid and reproducible and also economical infectivity assay for all the 4 types of DEN viruses. Since the introduction of cell culture systems, many people have developed various assay systems for DEN viruses. The principle of these methods is either by plaque assay (Schulze and Schlesinger, 1963; Miles and Austin, 1963; Georgiades *et al.,* 1965; Hotta *et al.,* 1966; Westaway, 1966; Russell *et al.,* 1967; Bergold and Mazzali, 1968; Banerjee, 1969; Yunker and Cory, 1975) with its adaptation to micromethod (Skuhavachana *et al.,* 1969; DeMadrid and Porterfield, 1969; Fujita *et al.,* 1975) or to hemadsorption negative plaque (Makino and Mifune, 1975), or by interference method (Halstead *et al.,* 1964). We have developed the focus counting method for the rapid titration of DEN viruses (Igarashi and Mantani, 1974; Okuno *et al.,* 1977, 1978). As shown in this paper, this method has almost the same sensitivity as the standard SMB–LD_{50}, or PFU assay on LLC–MK_2 except DEN–2. The principle of focus assay was originally used for rabies virus (Smith *et al.,* 1973) and recently for rapid detection of poliovirus (Katzenelson, 1976). The method was used for the

cloning of DEN virus which did not form plaques (Lubinski *et al.,* 1973). As already discussed (Igarashi and Mantani, 1974), this method has several advantages to give rapid results using comparatively small amounts of cells and medium and comparatively small space for incubation.

The application of focus counting method for the N test (FR_{50}) also gave quite satisfactory results with comparable sensitivity as PR_{50} titer by plaque reduction (Russell *et al.,* 1967). Although microplaque methods were reported for dengue N tests (Sukhavachana *et al.,* 1969; Kanamitsu *et al.,* 1973; DeMadrid and Porterfield, 1974; Fujita *et al.,* 1975), which give fairly rapid results, the numbers of plaques observed in a single microtiter well are rather limited in contrast to our focus counting which could give a good dose-response up to 200 foci/well. The practical application of the FR_{50} assay has already been reported (Quina *et al.,* 1978) and now in this paper. Compared with HI test, N test is more sensitive to detect type-specific antibodies as has been described for JE virus (Kobayashi *et al.,* 1967; Minamitani *et al.,* 1974; Fukunaga *et al.,* 1974a), and also is more type-specific than HI. Considering the possibilities of multiple flavivirus infections prevalent in Southeast Asia, N test is very important to detect type-specific antibodies in the serodiagnosis and seroepidemiology of dengue infections in this area (Kanamitsu *et al.,* 1973).

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フォーカス計数法によるデングウイルス感染価測定と中和抗体測定法の比較的感度

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4つの異なる血清型のデングウイルスの感染価を BHK-21 細胞でのフォーカス計数法 (FFU), LLC-MK2 細胞でのプラック形成 (PFU), および乳呑マウス脳内接種による LD_{50} によって測定した。すべての型のデングウイルスについて FFU は LD_{50} とほぼ同じ値であり, 2型以外は PFU ともほぼ同じであった。2型では PFU は FFU 又は LD_{50} よりも低値を示した。デングウイルスの各型に対する抗血清の同型ウイルスに対する中和抗体価を50%フォーカス減少法 (FR_{50}) と 50%プラック減少法 (PR_{50}) で求めた。2型以外の抗血清では FR_{50} と PR_{50} はほぼ同じ値であったが, 2型では PR_{50} が FR_{50} よりも高い値を示した。この方法は, また, 日本脳炎ウイルスおよびチクングニアウイルスにも応用できた。数名のヒト血清の抗体測定から, 中和抗体測定は血球凝集抑制抗体測定よりも一般に感度が良く, 型特異的である事が示された。

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SUSCEPTIBILITY OF VARIOUS CELL LINES TO RABIES VIRUS

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Abstract: Twelve established cell lines including 7 mouse-derived cell lines and primary chick embryo fibroblast cells were examined for their susceptibility to rabies virus. The results indicated that, in addition to CER cells and BHK–21 cells, murine neuroblastoma cells and human neuroblastoma cells (SYM cells) are highly permissive to infection with both fixed and field rabies strains and suggested the usefulness of these neuronal cell lines in various immunologic aspects of rabies studies in which histocompatibility requirements play a significant role.

INTRODUCTION

Rabies virus has been successfully propagated in many tissue culture systems in the past (Kissling, 1958; Wiktor *et al.*, 1964; Kondo, 1965; Diamond, 1967; Hummeler *et al.*, 1967; Sedwick and Wiktor, 1967; Wiktor *et al.*, 1969). These include primary cell cultures such as hamster kidney cells and chick embryo fibroblast cells, and human diploid cells which have been used mainly for the purpose of vaccine production (Kissling, 1958; Wiktor *et al.*, 1964; Kondo, 1965; Wiktor *et al.*, 1969; Grandien, 1977). Of established cell lines, BHK–21 cells and their sublines have been used by several investigators for the production of high yields of virus (Sokol *et al.*, 1968, 1969; Schneider *et al.*, 1971; Cox *et al.*, 1977; Kawai and Matsumoto, 1977). CER cells, recently established by Dr. Motohashi, Japan, have also been demonstrated to be highly permissive to rabies virus infection and to have a potential for isolation of field strains of rabies virus (Smith *et al.*, 1977, 1978).

One aspect of the search for susceptible cells in tissue culture systems is to develop appropriate tissue culture cells for use in *in vitro* tests of immunological responsiveness to rabies in laboratory animals and in human. In these sorts of studies, it has been demonstrated that a histocompatible system (H–2 in the mouse) is required for the full expression of the interaction between immune lymphocytes and the virus-infected target cells (Doherty and Zinkervagel, 1975). The present study was initiated to examine the susceptibility of mouse-derived cell lines to rabies virus, with the ultimate goal of studying the immunological responses to the virus in mice. In addition, some commonly used cell types derived from other animals were simultaneously tested for permissiveness to the virus.

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Materials and Methods

Virus: Three strains of rabies virus were used. The HEP-Flury strain of rabies virus was supplied by Dr. Kondo, National Institute of Health, Tokyo, Japan, and used at passage levels of 106 to 111 in chick embryo fibroblast cells. HEP-Flury of 2nd to 3rd passage level in CER cells was used in some experiments. The mouse brain-adapted CVS strain was used as a 10 per cent infected mouse brain suspension at the 56th passage level. In some experiments, the CVS strain, plaque-cloned in Vero cells (Buckley and Tignor, 1975) obtained from Yale Arbovirus Research Unit (YARU), Yale University, was used after one additional passage in murine neuroblastoma cells. The 1088 rabies strain, originally isolated from a woodchuck by the Center for Disease Control, U.S.A., was obtained from YARU and used at the 3rd passage level in suckling mouse brain.

Cells and culture media: Seven mouse-derived cell lines and 6 cell cultures derived from other animals (5 continuous lines and primary chick embryo fibroblasts) were tested for their susceptibility to rabies virus. Growth media for these cells were shown in Table 1. After virus infection, the percentage of heat-inactivated fetal calf serum was reduced to 2 or 5 per cent.

Table 1 Cell lines tested for susceptibility to rabies virus

Mouse-derived		Other animal-derived	
Cell	Growth medium	Cell	Growth medium
L	Eagle MEM+10% FCS[a]	Vero	Eagle MEM+10% FCS
L 1210	RPMI 1640 +20% FCS	BSC–1	Eagle MEM+10% FCS
L 5178Y	RPMI 1640 +20% FCS	BHK–21	Eagle MEM-Hanks salts +10% FCS
Neuroblastoma (N–18)	Dulbecco's modified Eagle MEM+10% FCS	CER	Eagle MEM+10% FCS
SR–CDF1–DBT[b] (glioma)	Eagle MEM+10% FCS	SYM[c] (Human neuroblastoma)	1/2 Eagle MEM +1/2 RPMI 1640 +20% FCS
Mastocytoma (P–815)	Eagle MEM+10% FCS	CEF[d]	Eagle MEM-Hanks salts +10% FCS
Ehrlich ascites tumor	Eagle MEM+10% FCS		

a) Heat-inactivated fetal calf serum. All culture media contained antibiotics.
b) Established by Dr. T. Kumanishi (1967), Tokyo University, Japan.
c) Established by Dr. M. Sekiguchi (personal communication), Tokyo University, Japan.
d) Primary chick embryo fibroblast cells.

Parameters of susceptibility: Infection of the cells with virus was carried out in the presence of 50 μg/m*l* of DEAE-dextran for 1 h at 37 C. Infected cultures were then refed with maintenance media or overlay media and incubated at 37 C. At least 2 tests using the following parameters were performed to examine permissiveness.

a) *Fluorescent antibody staining.* The cells capable of adhering to glass were cultured in 4-chamber Lab-Tek chamber slides (Lab-Tek Products, Miles Laboratories, Naperville, Ill.) and infected at a multiplicity of infection (MOI) of approximately 0.1 focus forming unit (FFU)/cell and incubated in a 5% CO_2 incubator. Other cells not capable of adhering to glass were packed by low speed centrifugation and infected at an MOI of 0.1 FFU/cell. The cells were then transferred to test tubes at appropriate cell densities and incubated.

On days 1, 2, 3 and 4 after infection, these cells were examined for cytoplasmic fluorescent antigens using anti-rabies virus FITC-labelled goat serum. The percentage of cells containing specific cytoplasmic fluorescent antigens was determined by examining at least 300 cells. The amount of cytoplasmic fluorescent antigens in the cells was estimated as large, intermediate and minute, respectively, depending on the relative size and distribution of inclusions in the cells.

b) *Cytopathic effect.* Monolayers in culture flasks and suspension cultures in test tubes, both infected with virus at a MOI of 0.1 FFU/cell, were observed for visible cytopathic effect under an inverted microscope for 8 days.

c) *Plaque formation.* The ability of some cells to support plaque formation was also tested in certain cell lines. The procedures were essentially the same as those described by Yoshino *et al.* (1966) except that calf serum and agar in the overlay were replaced by heat-inactivated fetal calf serum and 2% Sephadex G–200 (Schneider, 1973), respectively. Infected cultures were observed for plaques for 8 days.

d) *Hemadsorption-negative* (HAD^-) *plaque formation.* This was done as described by Makino and Mifune (1975) for dengue virus assay.

e) *Persistent infection.* To examine if the percentage of fluorescent antigen-positive cells increased during subcultures, the cultures infected with virus at a MOI of 0.1 FFU/cell were refed with growth medium and subcultured 4 times at an interval of 5 to 7 days with a split ratio of 1 to 4. At the time of subculture, the percentage of fluorescent antigen-positive cells in a culture was determined as described before. The cultures which contained no cells with cytoplasmic fluorescent antigens at the 2nd and the 4th subculture levels were considered to be "cured".

f) *Virus yield.* Monolayers of susceptible cells in culture flasks were infected with virus at a MOI of 0.1 FFU/cell and incubated for 4 days. The cultures were frozen and thawed 3 times and the supernatant was assayed for infectivity after low speed centrifugation. Infectivity of the virus was assayed in CER cells by the fluorescent focus formation method using 4-chamber Lab-Tek chamber slides as described by Smith *et al.* (1977).

Results

Of seven mouse-derived cell lines tested, neuroblastoma cells were most sensitive to all strains of rabies virus tested. By fluorescent antibody staining, almost all of the N18 cells contained large, widely spread fluorescent antigens by day 4 after infection even with a field rabies strain (1088), and persistent infection could be readily established. In contrast, SR–CDF1–DBT cells and Ehrlich ascites tumor cells were sensitive only to the tissue culture-passaged, attenuated HEP-Flury strain

of rabies virus. Susceptibility of other cell lines derived from the mouse was found to be very low (Table 2).

Table 2 Susceptibility of mouse-derived cell lines to rabies virus as determined by fluorescent antibody staining, CPE induction and ability to support virus growth during subculture

Cell		Virus strain		
		1088	CVS (mouse brain-passaged)	HEP-Flury
L	FA[a]	0–2[b] (M)[c]	0–2 (M)	0–2 (M)
	CPE[d]	—	—	—
	Persistent infection	cure	cure	cure
L 1210	FA	20–30 (M)	20–30 (M)	15–35 (M)
	CPE	—	—	—
	Persistent infection	cure	cure	cure
L 5178 Y	FA	10–20 (M)	10–20 (M)	10–20 (M)
	CPE	—	—	—
Neuroblastoma (N–18)	FA	95–100 (L)[e]	95–100 (L)	95–100 (L)
	CPE	—	±	±
	Persistent infection	yes	yes	yes
SR–CDF1–DBT (glioma)	FA	N.D.[g]	0–2 (M)	80–90 (I)[f]
	CPE		—	—
Mastocytoma (P–815)	FA	0–0.1 (M)	0–0.1 (M)	0–0.1 (M)
	CPE	—	—	—
Ehrlich ascites tumor	FA	N.D.	0–2 (M)	80–95 (L)
	CPE		—	—

a) Fluorescent antibody staining.
b) Per cent of fluorescent antigen containing cells on day 4 after infection in 3 experiments.
c) Minute, pinpoint like antigen.
d) Cytopathic effect.
e) Large, widely spread antigen masses in the cells.
f) Intermediate antigen.
g) Not done.

Cell lines derived from other animals appeared, in general, to be more permissive to rabies virus infection than were the mouse-derived cells, and it was difficult to compare the susceptibility to rabies virus among these cell lines. CER and SYM cells, however, had the capacity to replicate the 1088 strain to almost the same degree as the fixed rabies strains as determined by fluorescent antibody staining, and the infection of CER cells could be successfully maintained in serial subcultures (Table 3). Vero and BSC–1 cells were sensitive only to fixed rabies strains (CVS and HEP-Flury strains) by fluorescent antibody staining but produced lower yields of the virus as

Table 3 Susceptibility of non-mouse-derived cell lines to rabies virus as determined by fluorescent antibody staining, CPE induction, plaque and HAD⁻ plaque formation and ability to support virus growth during subculture

Cell		Virus strain		
		1088	CVS (mouse brain-passaged)	HEP-Flury
Vero	FA[a]	5–10[b] (M)[c]	80–90 (I)[d]	80–90 (I)
	CPE	−	+	+
	Plaque	−	+	−
	Persistent infection	cure	yes	yes
BSC–1	FA	5–10 (M)	50–75 (I)	50–75 (I)
	CPE	−	−	−
	HAD⁻ plaque	−	+	+
BHK–21	FA	20–25 (I)	95–100 (L)[e]	95–100 (L)
	CPE	−	+	±
	Persistent infection	yes	yes	yes
CER	FA	50–100 (I)	95–100 (L)	95–100 (L)
	CPE	−	+	−
	Plaque	−	+	−
	HAD⁻ plaque	−	+	+
	Persistent infection	yes	yes	yes
SYM (Human neuroblastoma)	FA	65–75 (I)	95–100 (L)	95–100 (L)
	CPE	−	±	±
CEF[f]	FA		30–50 (I)	95–100 (L)
	CPE	N.D.[g]	−	±
	Plaque		−	+

a) Fluorescent antibody staining.
b) Per cent of fluorescent antigen containing cells on day 4 after infection in 3 experiments.
c) Minute, pinpoint like antigen.
d) Intermediate antigen.
e) Large, widely spread antigen masses in the cells.
f) Primary chick embryo fibroblast cells.
g) Not done.

compared with those of CER and BHK–21 cells (Table 4). BSC–1 cells were shown to be useful for the infectivity assay of fixed strains of rabies virus. Vero cells and CER cells permitted plaque formation by the CVS strain of rabies virus.

Virus yields from a limited number of susceptible cell lines are shown in Table 4. In general, tissue culture-passaged rabies virus grew to higher titers than did mouse brain-passaged rabies virus. Among these cell lines, murine neuroblastoma cells and CER cells supported replication of tissue culture-passaged CVS rabies to the highest titers. The highest yields of HEP-Flury virus were obtained in CER cells and CEF cells.

Table 4 Rabies virus yields from susceptible cell lines[a)]

Cell	Virus strain		
	CVS (mouse brain-passaged)	CVS (plaque-purified murine neuroblastoma-passaged)	HEP-Flury
Murine neuroblastoma (N–18)	5.3[b)]	8.0	5.9
Vero	3.6	6.4	5.8
BSC–1	3.2	N.D.[c)]	5.0
BHK–21	5.0	7.0	6.3
SYM (Human neuroblastoma)	5.5	7.2	N.D.
CER	5.7	7.7	7.3
CEF[d)]	N.D.	N.D.	7.3

a) Monolayers in culture flasks were infected with virus at an MOI of 0.1 FFU/cell and incubated at 37 C for 4 days. The cultures were frozen and thawed 3 times and supernatant fluid was assayed for infectivity after low speed centrifugation.

b) Log_{10} FFU/m*l*.

c) Not done.

d) Primary chick embryo fibroblast cells.

Discussion

Murine neuroblastoma cells, which were derived from an A strain mouse and which express some neuronal characteristics in culture, were demonstrated to have the greatest susceptibility to rabies of the mouse-derived cell lines tested in this study. Since these cells permit expression of antigen on the surface after infection with both fixed and street rabies virus, they have been recently chosen as target cells by us (Mifune and Tignor, in press) and others (Wiktor *et al.*, 1977) in the *in vitro* studies of immunological responses of rabies in mice. In addition, these cells have potential for isolation of field rabies strains (Smith *et al.*, 1978) and provide a system in which to examine the interaction between field rabies strains and neuronal cells.

The susceptibility of L cells and mastocytoma cells derived from C_3H mouse and DBA mouse, respectively, was shown to be very low. The high susceptibility of CER cells to rabies virus including field strains was first described by Smith *et al.* (1977). In this study, further and comparative observations were made with some other cell lines regarding their susceptibility to the virus. The results showed that, in addition to CER and BHK–21 cells, SYM cells derived from a human neuroblastoma are highly susceptible to both fixed and field rabies strains and might be a useful cell line for the immunological studies of human rabies which might require a histocompatible system in the assays.

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狂犬病ウイルスに対する種々細胞株の感受性

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7株のマウス由来組織培養細胞株を含む12の細胞株と初代鶏胎児細胞について，狂犬病ウイルスに対する感受性を検討した結果，これまで報告のあったCER細胞ならびにBHK-21細胞の他に，マウス神経芽細胞腫およびヒト神経芽細胞腫（SYM細胞）由来の細胞が，狂犬病ウイルスの固定毒ならびに街上毒に高い感受性を示し，マウスおよびヒトにおける狂犬病の研究，特に，組織適合抗原の適合性が重要な免疫学的研究に，これらの細胞が非常に有用であることが示唆された。

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3 文献請求先：大分医科大学微生物学講座（昭和55年以降）

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THE EPIDEMIOLOGY OF MEASLES (RUBEOLA)

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There are important differences between the epidermiological features of measles in developed countries (e.g. Japan) and those in developing countries. In Japan, for example, measles is a disease of nursery school and kindergarten children (Figure 1). It follows a 2–4 year cycle and has a seasonal distribution (Figure 2). Its clinical couse is mild and its fatality rate is very low (0.015% in 1975). This picture is very different from that of West Africa where measles is one of the most serious and feared diseases of childhood. The attack rate is very high and the median age of the disease is about 18–24 months (Figure 3). The disease occurs throughout the year with peaks during the dry months of November to May (Figure 4). The disease is often accompanied by one or more of the following complications: stomatitis, purulent conjunctivitis, otitis media, diarrhoea and bronchopneumonia. Many children die from measles and others are disabled through blindness or deafness. The general fatality rate is about 2–5 per cent, but higher rates (20% or more) have been recorded among hospital cases (Morley, 1969).

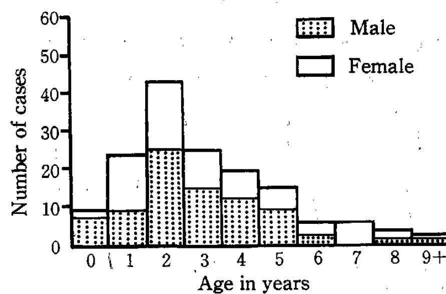

Figure 1 Cases of Measles reported in the Wadayama district (rural Japan) 1977. — Age/sex distribution.

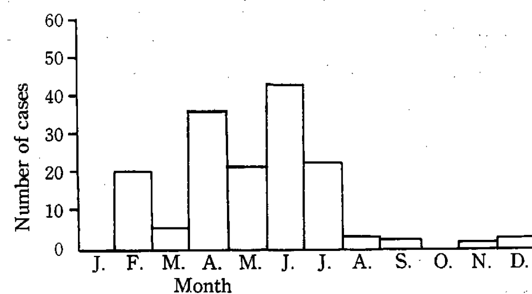

Figure 2 Cases of Measles reported in Wadayama district (rural Japan) 1977. — Monthly distribution.

The difference between measles in developed countries and that in developing countries is due to a combination of many factors which include malnutrition, low standard of living and cultural practices. In many of the developing countries, malnutrition is very prevalent among preschool children, and measles is severe in the malnourished. Besides, measles itself can precipitate malnutrition in borderline cases. In West Africa, it is customary for mothers to carry their infants on their

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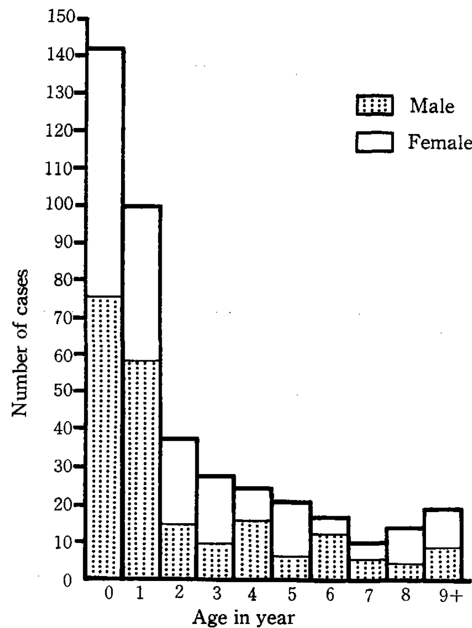

Figure 3 Cases of Measles reported in the Akosombo district (rural Ghana) 1977. — Age/sex distribution.

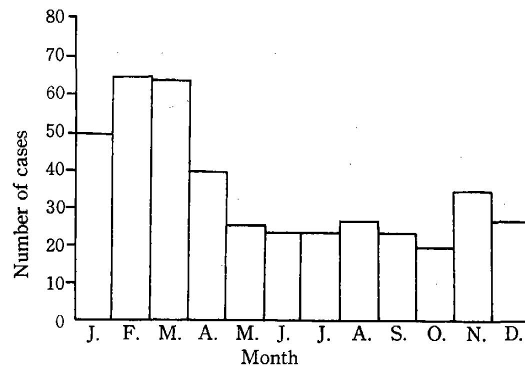

Figure 4 Cases of Measles reported in the Akosombo district (rural Ghana) 1977. — Monthly distribution.

backs. This means that a child goes everywhere (markets, festivals and other gatherings) with mother. This may start as early as the second week of life. Children are thus exposed to measles and other droplet infections very early in life and they are, therefore, liable to be attacked by measles as soon as maternal antibodies disappear at about the age of six months. Furthermore, the adverse effects of the disease are enhanced by traditional beliefs and practices. Sometimes food is withheld or purgatives are given "to bring out the rash", because it is believed that measles rash on the body is less dangerous than rash on the intestinal lining. The child may not be bathed for days, and instead, it is plastered with local mixtures again in an attempt to bring out the rash. Other important factors in the pathogenesis of measles in West Africa include the low standard of living and the lack of adequate medical care, especially in rural areas. All the above combine to make measles a very serious and important disease in many developing countries. From 1966 to 1972, a campaign against measles using vaccination was conducted in West Africa as part of the worldwide smallpox eradication programme. West Africa was declared free from smallpox in 1975, but no lasting impression was made on measles. The prevention and control of measles are, therefore, among the urgent challenges of tropical medicine today.

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