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Experimental Infection of the Korean Chipmunk (*Tamias sibiricus asiaticus*, Gmelin) with *M. leprae*[1,2]

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Shepard's observations ([18, 20]) that inoculation of *Mycobacterium leprae* into foot pads of mice resulted in a consistent but limited growth of the organisms have been amply confirmed and extended ([16, 17, 19]). Though some features of generalized infection of mice with *M. leprae* have been observed in the thymectomized and irradiated mice ([17]), a definite ceiling effect became evident on the maximal number of *M. leprae* grown in the foot pads of immunologically competent mice ([17, 20]).

Since 1967 we have been working on the experimental infection of the Korean chipmunk (*Tamias sibiricus asiaticus*, Gmelin) with *M. leprae* in search for a better animal host in the study of human leprosy.

Application of Korean chipmunks as experimental animals for the growth of *M. leprae* is based on the facts that: 1) the chipmunks are readily available in large numbers and they can be easily maintained in the laboratory for experimental purpose, 2) the average life span of the chipmunks is known to be approximately three years which is longer than that of mice, and 3) the chipmunks proved to be highly sensitive to experimental infection with *M. tuberculosis* ([1]).

In this communication we present the evidences of active growth of *M. leprae* in foot pads and ears of the chipmunks, major characteristics of pathological changes in these inoculated tissues, and the result of clinical study in a series of leprosy patients with chipmunk lepromin antigen prepared from infected chipmunks' foot pads.

MATERIALS AND METHODS

Animals. Korean chipmunks of both sexes, age of less than one year and 50 gm to 70 gm of body weight were used throughout the experiments. At the laboratory they were maintained in metal rabbit cages (eight to ten chipmunks per cage) and fed with boiled corn, chestnuts, acorn, boiled pupae of silkworm, vegetable and water. Prior to inoculation with *M. leprae*, the chipmunks were allowed to become accustomed to laboratory maintenance for two to four weeks.

Mice of CFW strain, both sexes and weighing 18 ± 2 gm were also used. The temperature of the animal room was kept at $20 \pm 1°C$.

M. leprae. Biopsied nodules from fresh untreated lepromatous leprosy cases were the source of *M. leprae* for animal inoculation. The nodules were used either immediately after biopsy or kept frozen at $-15°C$ for up to four weeks before use.

Two preparations of *M. leprae* were used for animal inoculation, the first one was prepared by conventional grinding method followed by light centrifugation, and the second by trypsin purification method as shown in Figure 1.

Counting of numbers of acid-fast bacilli (AFB). The pinhead method of Hanks ([3]) was used.

Lepromin antigens. The standard lepromin antigen was prepared from biopsied lepromatous nodules and the chipmunk lepromin antigen from infected foot pads of the chipmunks by the method recommended by Hanks ([2]), and each of the antigen preparations contained 160×10^6 AFB per ml.

Pathologic preparations. The infected tissues were fixed in buffered formalin, paraffin embedded, sectioned and stained with hematoxylin-eosin (H-E) and acid-fast (AF) stains.

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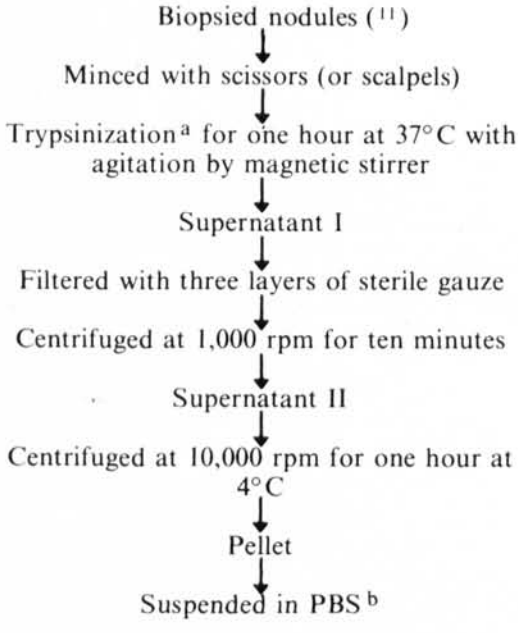


[a] Trypsin solution; 0.25% in PBS
[b] PBS (phosphate buffered saline, Dulbecco)

FIG. 1. Trypsin purification of *M. leprae* from biopsied lepromatous nodules.

RESULTS

Preparation of trypsin-purified M. leprae from biopsied nodules. Trypsin purification method (Fig. 1.) resulted in a good yield of rather well-dispersed homogeneous populations of *M. leprae* from biopsied nodules with a negligible contamination of tissue debris. Additional advantages of this purification method are as follows: 1) it completely omits conventional grinding or homogenization of lepromatous tissues which have been routinely applied in the collection of *M. leprae* from biopsied nodules, 2) this method allows the preparation of a concentrated suspension of semipurified *M. leprae* by simply adjusting the volume of suspending PBS to the pellet obtained by centrifugation at 10,000 rpm for one hour at 4°C.

Growth of conventionally collected M. leprae in Korean chipmunks. In the first series of experiment, animal inoculations were made with *M. leprae* prepared by conventional grinding method. Over 30 chipmunks were inoculated with *M. leprae* into the left hind foot pads, and the inoculum contained 1.0×10^6 AFB in a volume of 0.05 ml per foot pad.

Following the inoculation, chipmunks were sacrificed at given intervals and the total numbers of AFB per inoculated foot pad were determined. Growth pattern of *M. leprae* observed in this experiment is shown in Figure 2.

Significant increases in the total numbers of AFB per inoculated foot pad became apparent after a lag phase of approximately seven months following the inoculation. During the lag phase, the numbers of AFB per inoculated foot pad remained either close to or below the level of the base-line which represented the minimal number of AFB detected by the pinhead method.

Of three chipmunks sacrificed at 7.5 months post-inoculation, the total number of AFB contained in the foot pad of one chipmunk was 2.2×10^7, and this preparation of *M. leprae* was used for the second passage experiment and the results are shown in Table 1.

As in the first passage experiment, apparent increases in the total numbers of AFB per inoculated foot pad were observed at ten months following the inoculation.

The morphology of AFB at the time of growth of *M. leprae* in the chipmunks' foot

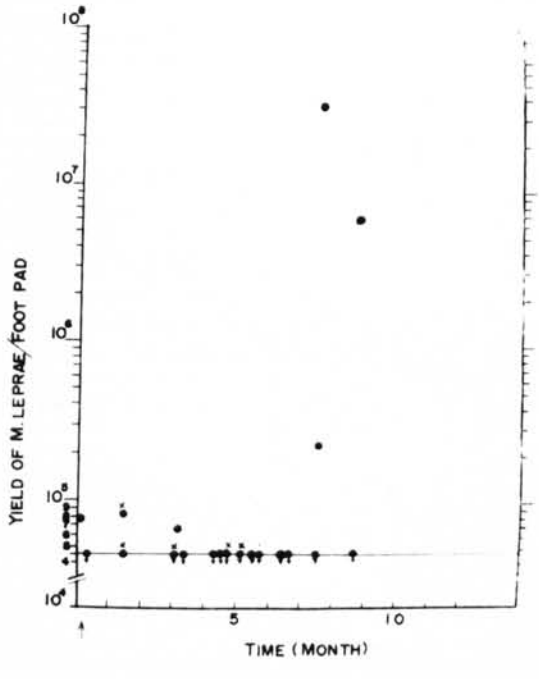


FIG. 2. Growth of *M. leprae* in foot pads of the chipmunks.

TABLE 1. *Result of the second passage of* M. leprae *in the chipmunks' foot pads.*

Inoculum (per foot pad)	No. of AFB/foot pad	
	4.5 mo.	10 mo.
1.0×10^5	—[a]	3.7×10^6
	—	6.9×10^5

[a] Not detected by the pinhead method.

pads appeared to fit well with the description of solid-form bacilli, and the subpassage experiment substantiated the viability of those AFB collected during the period of active growth of *M. leprae* in the inoculated tissues. The third passage experiment is now in progress.

In parallel with experimentation in the chipmunks, the same preparation of *M. leprae* (conventionally collected) was inoculated into foot pads of mice (1.0×10^5 AFB/foot pad), and the growth curve was followed. As reported by others ([17, 18, 20]), the growth of *M. leprae* occurred after a lag period of about seven months following the inoculation, but the maximal yield of AFB per inoculated foot pad remained close to the level of 10^6 as shown in Figure 3.

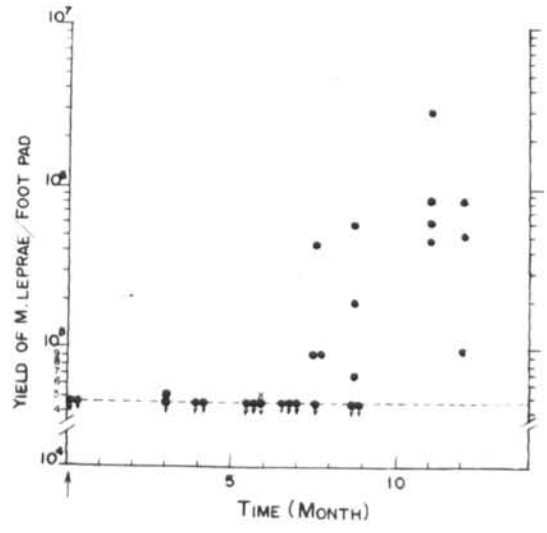


FIG. 3. Growth of *M. leprae* in foot pads of mouse.

Growth of trypsin-purified M. leprae in foot pads and ears of Korean chipmunks. The viability of trypsin-purified *M. leprae* was tested by inoculating into foot pads and ears of the chipmunks. The inoculum containing 1.0×10^5 AFB in a volume of 0.05 ml was injected into left hind foot pads or left ear, and the heat-killed (70°C for one hour) preparation of trypsin-purified *M. leprae* was inoculated into right hind foot pads or right ears as controls. The result of counting of total AFB in the foot pads or ears of the chipmunks at two weeks and at 8 and 12 months showed definite increases in the total numbers of AFB per inoculated tissue 8 and 12 months post-inoculation (Table 2).

The general pattern of multiplication of trypsin-purified *M. leprae* in the chipmunks' foot pads or ears appeared to be similar to those of conventionally collected *M. leprae* in the same animals. Neither foot pads nor ears inoculated with heat-killed *M. leprae* contained detectable numbers of AFB throughout the experimental period. This experiment established that the trypsin purification method resulted in the collection of a semi-purified preparation of viable *M. leprae*. Based on this observation further studies of animal inoculation were made with trypsin-purified *M. leprae*.

Pathologic changes in the inoculated tissues. During the early phase of multiplication of *M. leprae* (conventionally collected or trypsin-purified) in foot pads or ears of the chipmunks, no specific pathologic changes were observed. However, when the inoculated chipmunks were maintained for a longer period of time, characteristic leprotic changes became manifest, such as extensive leproma formation (Fig. 4.), presence of massive numbers of AFB in the leproma cells (Fig. 5.) and the evident involvement of dermal nerve fibers by AFB (Fig. 6.).

The lesions exhibited characteristic features of lepromatous leprosy in human beings, and morphologically the leproma cells represented typical macrophages of which the cytoplasm was filled with large numbers of AFB (Fig. 5).

Preparation of the chipmunk lepromin antigen. In the next experiment we attempted to prepare chipmunk lepromin antigen with AFB harvested from infected tissues of the chipmunks. For this purpose about 50 chipmunks were inoculated in both hind foot pads or both ears with an inoculum containing 1.0×10^6 trypsin-purified *M. leprae*. The animals were sacrificed ten months post-inoculation for the harvest. A

TABLE 2. *Result of inoculation of trypsin-purified* M. leprae *into Korean chipmunks.*

Experimental group	Site of inoculation		No. of AFB/foot pad or ear		
			2 wk	at 8 mo	at 12 mo
C	Ear lobe	L[b]	—[a]	1.8×10^5	5.7×10^6
			—	8.4×10^5	1.1×10^5
				—	9.5×10^4
				—	—
		R[c]	—	—	—
			—	—	—
D	Foot pad	L	—	2.9×10^5	1.9×10^5
			—	1.3×10^5	1.5×10^5
				—	5.6×10^4
				—	—
		R	—	—	—
			—	—	—

[a] Undetected by the pinhead method.
[b] L (left); inoculated with unheated *M. leprae.*
[c] R (right); inoculated with heat-killed *M. leprae.*

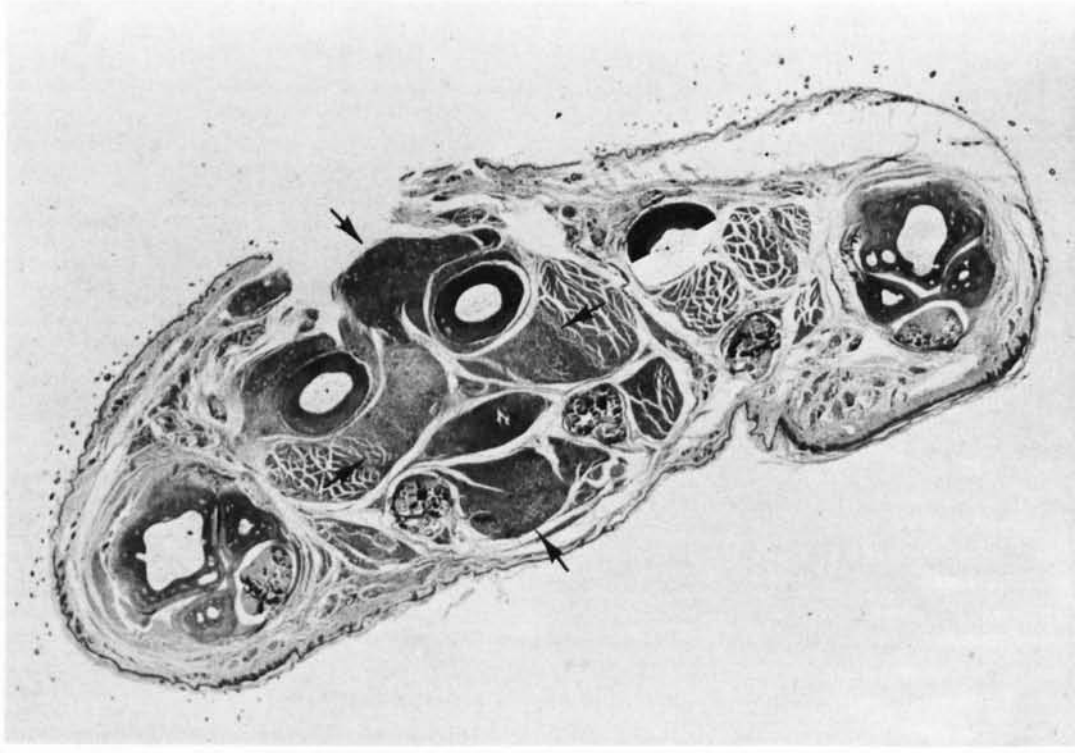


FIG. 4. Extensive leproma formation in the sole of foot pad of a chipmunk inoculated with trypsin-purified *M. leprae* 16 months previously. (H & E X 100) *Section and photo: ALM Leprosy Atelier.*

number of methods were applied for the collection of AFB such as conventional grinding, trypsinization and toluol extraction ([10]).

Among the methods employed (Tables 3 and 4), the trypsinization method (identical with the one illustrated in Fig. 1) gave the best result with respect to the number of AFB per ml and the total numbers of AFB harvested, but the final concentration of

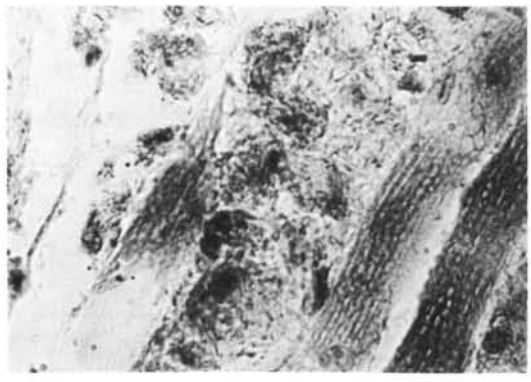


FIG. 5. Leproma cells containing a large number of acid-fast bacilli in muscle tissue of a chipmunk's foot pad inoculated with trypsin-purified *M. leprae* ten months previously. (A-F X 1,000)

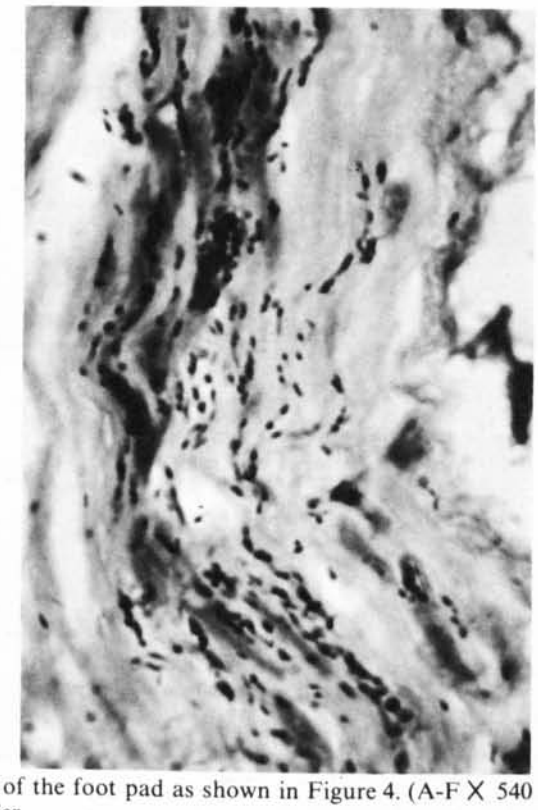
AFB was 1.9×10^7 per ml which was far smaller than the numbers of AFB contained in standard lepromin antigen (4).

However, in detailed examination of foot pads and ears of the chipmunks, inoculated with trypsin-purified *M. leprae* and sacrificed at ten months post-inoculation, we found swollen foot pads or ears in a considerable number of the chipmunks (Fig. 7). Such swollen foot pads or ears could be readily recognized by the naked eye, and the swollen foot pads consisted of packed masses of leproma cells which contained massive numbers of AFB (Fig. 8). Simple smears made from swollen infected tissues by a method comparable to Wade's skin scraping easily revealed the presence of enormous numbers of solid-form AFB and of numerous globi as shown in Figure 9.

Total numbers of AFB recovered from the two swollen foot pads (Fig. 7) were at the level of 2.0×10^{10} respectively (Table 5), and therefore the chipmunk lepromin antigen was prepared with AFB harvested from these swollen foot pads.

Skin tests with the chipmunk lepromin antigen. In a series of leprosy patients skin tests were conducted by inoculating intradermally 0.1 ml of the standard and of the chipmunk lepromin antigens and the readings of the tests were made at four weeks (the Mitsuda reactions) by the method recommended by Hanks *et al* (4).

As shown in Table 6, skin tests with the



FIGS. 6a, b. Acid-fast bacilli in a dermal nerve of the foot pad as shown in Figure 4. (A-F X 540 & 1,000). *Sections and photos: ALM Leprosy Atelier.*

TABLE 3. *Yields of AFB from infected tissues of the chipmunks by conventional grinding method.*

Infected tissues	No.[a]	Autoclaved or not	Yields	
			No. of AFB (/ml)	Total volume (ml)
Foot pad	10	Autoclaved	4.6×10^4	4.1
Ear	10		9.8×10^7	18.0
Foot pad	10	Not	7.1×10^6	5.0
Ear	10	autoclaved	9.3×10^6	20.0

[a] Inoculated with trypsin-purified *M. leprae* ten months previously.

TABLE 4. *Yields of AFB from infected tissues of the chipmunks by trypsinization and by toluol extraction.*

No. of infected ears[a]	Method	Yields	
		No. of AFB (/ml)	Total volume (ml)
6	Trypsinization	1.9×10^7	6.0
	Grinding	1.3×10^6 (supernatant)	10.0
14	Centrifuge	1.0×10^7 (sediment)	1.0
	Toluol extraction	2.3×10^6	3.0

[a] Inoculated with trypsin-purified *M. leprae* ten months previously.

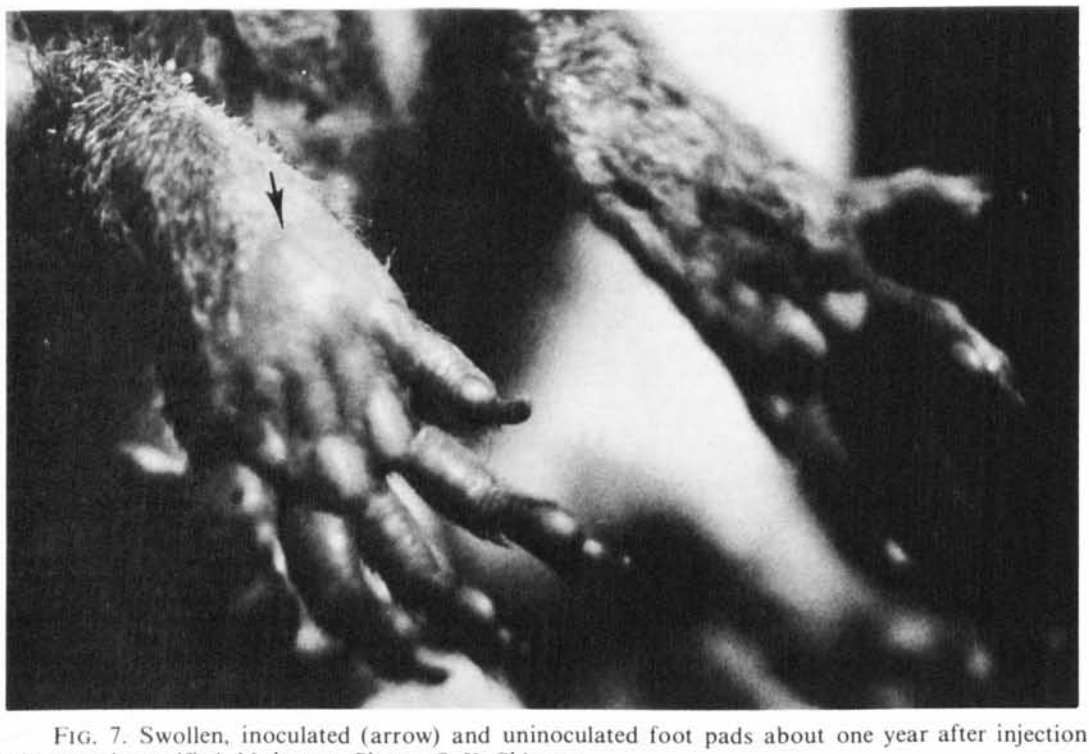


FIG. 7. Swollen, inoculated (arrow) and uninoculated foot pads about one year after injection with trypsin-purified *M. leprae*. *Photo: O. K. Skinsnes.*

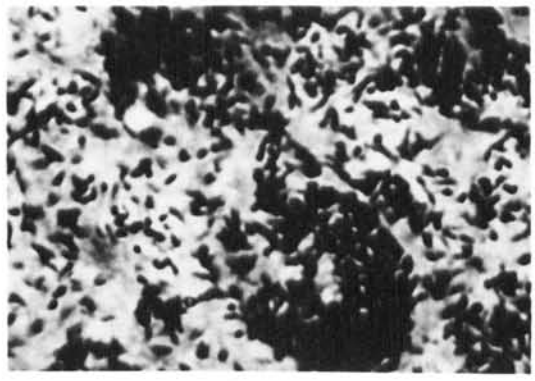


FIG. 8. Massive numbers of acid-fast bacilli in leproma cells of the swollen foot pad shown in Figure 4. (A-F X 1,000) *Section and photo: ALM Leprosy Atelier.*

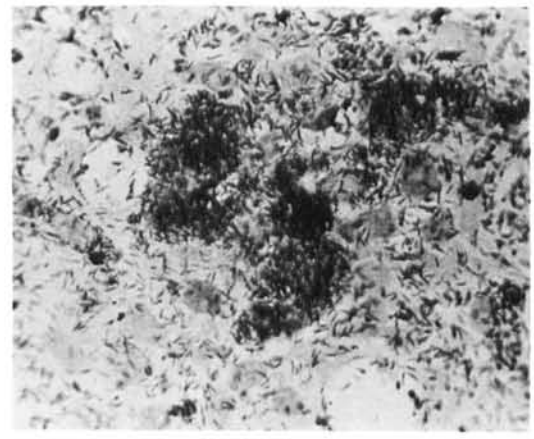


FIG. 9. Smear made from a swollen ear lobe of a chipmunk inoculated with trypsin-purified *M. leprae* ten months previously. (A-F X 1,000)

TABLE 5. *Yields of AFB from the swollen foot pads of chipmunks.*[a]

Specimen no.	Wet weight	No. of AFB / ml	Total volume	Total no. of AFB harvested
1	100 mg	3.4×10^9	5.9 ml	2.0×10^{10}
2	100 mg	3.6×10^9	5.9 ml	2.1×10^{10}

[a] Inoculated with trypsin-purified *M. leprae* ten months previously.

TABLE 6. *Result of chipmunk lepromin test in leprosy patients.*

OPD no.	Name	Age	Sex	Type of disease	Age at onset of disease	Length of treatment (years)	Lepromin test[a]		
							Previous test	Standard lepromin	C-lepromin
2010	H.D.P.	37	M	L	23	11	—	0	0
3248	K.J.L.	41	M	L	30	9	—	ND	0
3518	D.W.H.	21	F	L	11	8	—	±	±
4294	Y.W.K.	46	M	L	38	5	—	ND	0
4846	W.I.W.	32	M	L	20	2	—	ND	0
5025	N.K.O.	17	M	L	11	1	—	±	±
5066	C.S.C.	26	M	L	21	1	—	ND	0
5137	H.J.L.	44	M	L	37	6	—	ND	0
1565	I.K.L.	37	M	T	19	10	±	±	±
2069	W.K.K.	32	F	T	20	10	+	2+	3+
3728	S.Y.K.	39	M	T	29	7	+++	ND	3+u
4155	S.H.S.	26	M	T	20	6	+	2+	2+
4224	I.Y.K.	23	M	T	13	5	+	3+u	3+u
4731	H.S.W.	63	F	T	52	3	?	3+	3+
1341	C.S.K.	31	M	B	18	10	?	0	0
5107	J.Y.K.	25	M	B	23	1	+	ND	+
762′ sf	O.S.K.	14	M	Contact	—	—	ND	ND	+
4620′ sf	I.S.C.	33	M	Contact	—	—	ND	2+	2+

[a] Lepromin test:

C-lepromin: chipmunk lepromin.

Previous test: made with standard lepromin at the time of initial visit, and the reading of the results was based on the criteria of Madrid Congress, 1953.

Standard lepromin and C-lepromin: the reading of the result was based on the criteria proposed by Hanks *et al* [Bull. WHO **42** (1970) 703]. The eponential "u" signifies ulceration.

chipmunk lepromin antigen gave identical results to those of the standard lepromin antigen which was prepared from human lepromatous nodules. Skin test antigen similarly prepared from foot pad tissues of normal chipmunks failed to invoke any detectable tissue response at the site of the inoculation.

DISCUSSION

Results reported in this communication strongly indicate that Korean chipmunks are highly susceptible to infection with *M. leprae* and may provide a model for animal experimentation in the study of human leprosy.

Evidences for active growth of *M. leprae* in foot pads and ears of the chipmunks are as follows: 1) significant increases in total numbers of AFB per inoculated tissue occurred both in the first and second passage experiments after a lag phase of about seven months following inoculation; 2) pathologic involvement in the inoculated tissues exhibited characteristic leprotic changes such as extensive leproma formation, presence of massive numbers of AFB in the leproma cells (macrophages) and the involvement of dermal nerve fibers by AFB; and 3) total numbers of AFB harvested from the two swollen foot pads of the chipmunks inoculated with 10^6 of trypsin-purified *M. leprae* ten months previously were at the level of 2.0×10^{10} respectively, and these signified a net increase of over 10^4-fold in the total numbers of AFB. These findings are in sharp contrast to the ones reported by Nakamura and Hisai [14] in which only a limited multiplication of *M. leprae* was observed in the chipmunks' foot pads, and the bacillary counts of the chipmunks were less than those in mice.

The following observations seem to establish that the acid-fast organisms grown in the chipmunk tissues (foot pads and ears) are *M. leprae:* 1) the AFB used for animal inoculations were collected from biopsied lepromatous nodules, 2) the result of mouse foot pad inoculation of the same preparation of *M. leprae* (by conventional grinding method) was similar to the growth pattern of *M. leprae* in the mouse as reported by others [17, 18], 3) characteristic leprotic changes were noted in the infected tissues of the chipmunks and these changes exhibited pathologic features of lepromatous type leprosy in human beings, 4) skin tests in a series of leprosy patients with the chipmunk lepromin antigen prepared from the swollen infected chipmunks' foot pads gave identical Mitsuda reactions to those of the standard lepromin antigen prepared from biopsied lepromatous nodules. Furthermore, reports on the failure of growth of *M. lepraemurium* in foot pads of the chipmunks [8, 14] suffice to exclude the possibility that *M. lepraemurium* was involved in the growth of *M. leprae* observed in foot pads of the chipmunks in our experiments.

Except for the first series of experiments, preparations of trypsin-purified *M. leprae* were exclusively used for animal inoculation studies. A unique feature of this purification method of *M. leprae* is the complete omission of conventional grinding or homogenization which have become a routine procedure in the collection of *M. leprae* (or *M. lepraemurium)* from infected materials. Trypsin and other proteolytic enzymes have been utilized in the purification of *M. lepraemurium* and *M. leprae,* and evidence does exist to indicate that such purification procedures employing proteolytic enzymes do not affect the viability of *M. lepraemurium* [5, 7, 12, 13, 15]. Our study showed clearly that *M. leprae* purified from minced lepromatous tissues by the trypsinization method retained infectivity. The aims of trypsin purification of *M. leprae* from biopsied lepromatous nodules for animal inoculation are 1) the collection of viable homogeneous population of *M. leprae,* 2) the elimination of mechanical damages to *M. leprae* caused either by conventional grinding in mortar and pestle or by homogenization treatment of lepromatous tissues, and 3) the removal of contaminating tissue debris from *M. leprae* preparations.

One of the most significant findings in these chipmunk inoculation studies was the development of swellings of inoculated tissues (foot pads or ears) in a considerable number (7 out of 24 foot pads) of the chipmunks inoculated with trypsin-purified *M. leprae* ten months previously. Smears made from these swollen infected tissues by a method comparable to Wade's skin scraping easily revealed the massive growth of *M. leprae* in these tissues. In the mouse system such swellings of the inoculated tissues (foot

pads) were observed rarely in immuno-suppressed hosts ([17]). Furthermore, total numbers of AFB recovered from those swollen foot pads of the chipmunks were also highly significant with respect to the preparation of the chipmunk lepromin antigen for clinical use in human leprosy, and skin tests with the chipmunk lepromin antigen in a series of leprosy patients gave identical Mitsuda reactions to those of the standard lepromin antigen.

Recently, Kirchheimer and Storrs ([9, 10]) published preliminary results of experimental infection of armadillos with *M. leprae.* The data obtained from armadillo GSRI 8 appear to be quite impressive in connection with the features of systemic leprosy following inoculation into abdomen sites and ear lobes and of numbers of *M. leprae* per gram of autopsy materials.

In the mouse system, the phenomenon of generalized infection with *M. leprae* has been observed in immunosuppressed hosts ([17]) and in mice kept for about two years (full life span) after inoculation ([6]). At the present it is unknown whether such a generalized infection with *M. leprae* might occur in the chipmunk model following inoculation into either foot pad or ear. Studies are now in progress to observe the effect of long-term observation on the outcome of host-parasite interaction between chipmunks and *M. leprae.*

SUMMARY

1. *M. leprae,* obtained from lepromatous nodules either by conventional grinding or trypsin purification methods, multiplied in both foot pads and ears of the Korean chipmunks through the first and the second passage experiments. Growth of *M. leprae* in these inoculated tissues became evident after a lag phase of approximately seven months post-inoculation.

2. Characteristic leprotic changes were observed in foot pads of the chipmunks inoculated with trypsin-purified *M. leprae* 13 and 16 months previously, and these changes included extensive leproma formation, the presence of massive numbers of acid-fast bacilli in the foam cells and the involvement of dermal nerve fibers by acid-fast bacilli.

3. Among the chipmunks inoculated with trypsin-purified *M. leprae* for the preparation of the chipmunk lepromin antigen, apparent swelling of the inoculated tissues was observed in a considerable number of the chipmunks at ten months after inoculation. Two such swollen foot pads contained 2.0×10^{10} acid-fast bacilli each.

4. The results of skin tests in a series of leprosy patients with the chipmunk lepromin antigen, prepared with acid-fast bacilli harvested from swollen infected foot pads, were identical with those of standard lepromin antigen prepared from biopsied lepromatous nodules.

RESUMEN

1. *M. leprae* fue obtenido de nódulos lepromatosos mediante trituración convencional o por el método de purificación tripsínica y fue multiplicado tanto en el panículo adiposo como en el lóbulo auricular de ardillas Coreanas y a través de experimentos de primero y segundo pasajes. El desarrollo de *M. leprae* en estos tejidos inoculados fue observado después de una fase de retardo de alrededor de siete meses posteriores a la inoculación.

2. En el panículo podal de las ardillas se observaron los característicos cambios lepromatosos entre los 13 y los 16 meses posteriores a la inoculación de *M. leprae* tripsino-purificados. Estos cambios incluyeron una extensa formación de lepromas, la presencia masiva de bacilos ácido-resistentes en las células en espumadera como asi también la invasión de fibras neurales cutaneas por bacilos.

3. Entre las ardillas inoculadas con *M. leprae* tripsino-purificados para la preparación del antígeno lepromínico se observó una evidente tumefacción de los tejidos inoculados en un número considerable de animales a los 10 meses después de la inoculación. Dos de esos panículos podales contenían cada uno 2.0×10^{10} de bacilos ácido-resistentes.

4. En una serie de pacientes leprosos, los resultados de las pruebas cutaneas con antígeno lepromínico preparado con bacilos obtenidos de los panículos podales infectados fueron idénticos a los observados cuando el antígeno provino de biopsias de nódulos lepromatosos.

RÉSUMÉ

Chez des tamias coréens on a remarqué une prolifération bacillaire significative dans le coussinet plantaire et l'oreille après inoculation de *M. leprae* dans ces endroits. Les bacilles injectés ont été dérivés des lépromes soit par broyage au mortier soit par digestion avec trypsine. On a observé un temps de latence de croissance de *M. leprae* de sept mois environ. On a pu porter l'infection jusqu'au deuxième passage.

Les analyses histopathologiques des coussinets plantaires inoculés trieze et seize mois préalablement ont révélé les changements suivants: lépromes étendues, présence d'une quantité énorme de bacilles acido-résistants dans les cellules spumeuses et invasion des nerfs dermiques par des bacilles acido-résistants. Un total de 2.0×10^{10} bacilles acido-résistants par coussinet plantaire ont été trouvé dans deux animaux.

On a inoculé un groupe de tamias en vue de préparer une lépromine. Dans ce groupe d'animaux, chez un nombre considérable on a aperçu après 10 mois un gonflement des tissus inoculés. Une lépromine préparée des bacilles acido-résistants récoltés des coussinets plantaires gonflés a donné une lépromino-réaction identique à la réaction à la lépromine classique.

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