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Studies on the Pathogenicity of the ICRC Bacillus

Intratesticular Inoculation in Animals[1]

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Isolation, cultivation and growth characteristic studies on the ICRC bacillus have been reported in a series of publications since 1958([1, 2, 6]). Pathogenicity of the cultivated organism was first discussed in 1962 ([9]). The experimental work of ten years on human leprosy was later reviewed at a Ciba Foundation Study Group Meeting in London ([8]). The present report describes and discusses further studies on the pathogenicity of the ICRC bacillus on intratesticular inoculation in mice and hamsters.

MATERIAL AND METHODS

Bacilli. The ICRC bacilli were grown in the "modified fluid" (conditioned tissue culture medium) previously described ([2]). The bacilli were collected from 6-8 weeks' old cultures and washed with Tyrode's balanced salt solution, and the number of organisms was estimated roughly by hemocytometer ([2, 7]). Further centrifugation and necessary concentration of the bacillary suspension in balanced salt solution (BSS) gave approximately the dose used, viz., 1×10^8 bacilli in 0.01 ml.

Hamsters. Male golden hamsters (*Cricetus auratus*) weighing 50-60 gm. and also a few white (mutant) hamsters available in the colony, were used. A total of twenty hamsters were employed for the experiment.

Mice. Twenty dba(Bar) males and 20 hybrid (XVII × C57 Black) male mice weighing 20-22 gm. were used.

Methods of intratesticular inoculation. *Hamsters:* Hamsters were anesthetized with Nembutal (3 to 5 mgm. in saline solution, intraperitoneally). The testes were pushed into the scrotal sacs by thumb pressure on the abdomen. A rubber band was tied above the scrotal sac to hold the testes in place. With a tuberculin syringe it was possible to inject 0.05 ml. of bacillary suspension into each testis.

Mice: During initial trials it was observed that surgical exposure of the testis for injection insured correct lodgement of the bacillary material in the testicular tissue. Mice were anesthetized therefore with Nembutal (1 to 1.5 mgm. in normal saline solution per animal), and a small incision was made to open the abdominal cavity and expose the testes. The bacilli were then injected in the same way as in hamsters. Not more than 0.01 ml. of fluid could be injected per testis without force. The testis was then gently pushed back into the abdominal cavity and the incision closed with sutures.

A small group of animals, both hamsters and mice, were injected with Hyflosupercel (a substitute for silica) to lower body resistance to bacillary infection. The Hyflosupercel was suspended in normal saline solution and injected subcutaneously, 40 mgm. in hamsters and 20 mgm. in mice, before inoculation of the bacilli.

The experimental animals were observed daily. They were killed when they appeared weak, and tissues from visceral organs, testes, skin, foot pads and lymph nodes were removed and fixed in Zenker-formol solution.

Histopathology. The tissues were fixed in Zenker's fluid for 18 hours, washed under tap water overnight, dehydrated by an alcohol-butanol mixture ([4]) and embedded in paraffin. Sections six microns thick were cut and stained with Ziehl-Neelsen carbol-

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fuchsin by the Fite method ([5]) for the demonstration of acid-fast bacilli in tissues. Some tissues were fixed in 10 per cent neutral formalin for silver impregnation of nerves.

EXPERIMENTAL RESULTS

Experiment I. Intratesticular Inoculation in Hamsters

Macroscopic observations. Five months after inoculation of the bacilli all twenty hamsters showed enlargement of the testicular and/or epididymal mass. In some cases it was palpable. Some animals showed loss of hair from the skin on the scrotal sacs and two had swollen foot pads.

One animal died after 7 months and autopsy revealed an enlarged epididymis, about twice the size of the testis, and considerable testicular atrophy. The caudal end of the epididymis was enlarged in all the animals, with the formation of multiple nodules (Fig. 1). The inguinal lymph nodes were enlarged in most of the animals.

Microscopic observations. Table 1 presents detailed data on individual animals. (Three animals with postmortem changes are excluded from the table.)

Testis and epididymis. Testicular atrophy was rather common in this group of hamsters. Histiocytic granulomas brought about partial distortion of the seminiferous tubules in most of the animals. Only four animals had normal intact tubules, four had complete distortion, and the remaining twelve had partial distortion of tubules due to granulomas. Some testes displayed heavy histiocytic reaction in the testicular parenchyma (Fig. 2). The epididymis also displayed many focal granulomas full of acid-fast organisms, bringing about distortion of the organ to a varying degree, as noted in the tabulation.

Spleen and lymph nodes. The spleen and lymph nodes showed more or less similar reaction. Small groups of histiocytes with a few acid-fast bacilli were seen. In some lymph nodes the follicles were reduced in

TABLE 1. *Intratesticular inoculation of ICRC bacilli in hamsters.*

Animal No.	Postinoculation period in months	Qualitative index of histopathologic lesions			
		Testis	Epididymis	Spleen	Lymph node
1021[a]	6	—	3+	—	—
1045	6	2+	2+	2+	+
1066	7	2+	2+	2+	+
1016[a]	7	—	+	2+	2+
883	7	—	+	—	—
898	7	2+	—	+	+
974	8	+	+	—	—
899	8	+	+	—	+
1039	8	3+	2+	—	+
1044	8	3+	2+	—	—
1055	9	2+	+	—	+
1046	9	—	3+	—	+
1040	9	3+	2+	+	+
1047	12	2+	+	—	—
1054	14	2+	±	—	—
882	15	—	+	—	+
1022[a]	15	—	+	—	+
Total 17	—	11	16	5	11
Percentage	—	64	94	29	64

Note: Dose: 5×10^8 bacilli per testis approx. Age of animals: 1 to 4 months.

[a] Injected with silica prior to inoculation of bacilli.

\+ Indicates presence of acid-fast bacilli or focal granuloma.

2+ Few granulomata with acid-fast bacilli.

3+ Large leproma-like nodules: numerous acid-fast bacilli and disruption of histologic pattern of the organ to varying degree.

size, and the entire tissue appeared hyalinized.

Visceral organs. Only an occasional microscopic histiocytic granuloma was observed in the liver, in the capsule of the kidney, and on the abdominal walls. Acid-fast microorganisms were not abundant in these lesions.

Foot pads. Sections of swollen foot pads showed typical histiocytic granulomatous lesions with a few acid-fast bacilli (Fig. 3). The presence of fuchsinophil cells (Fig. 4)

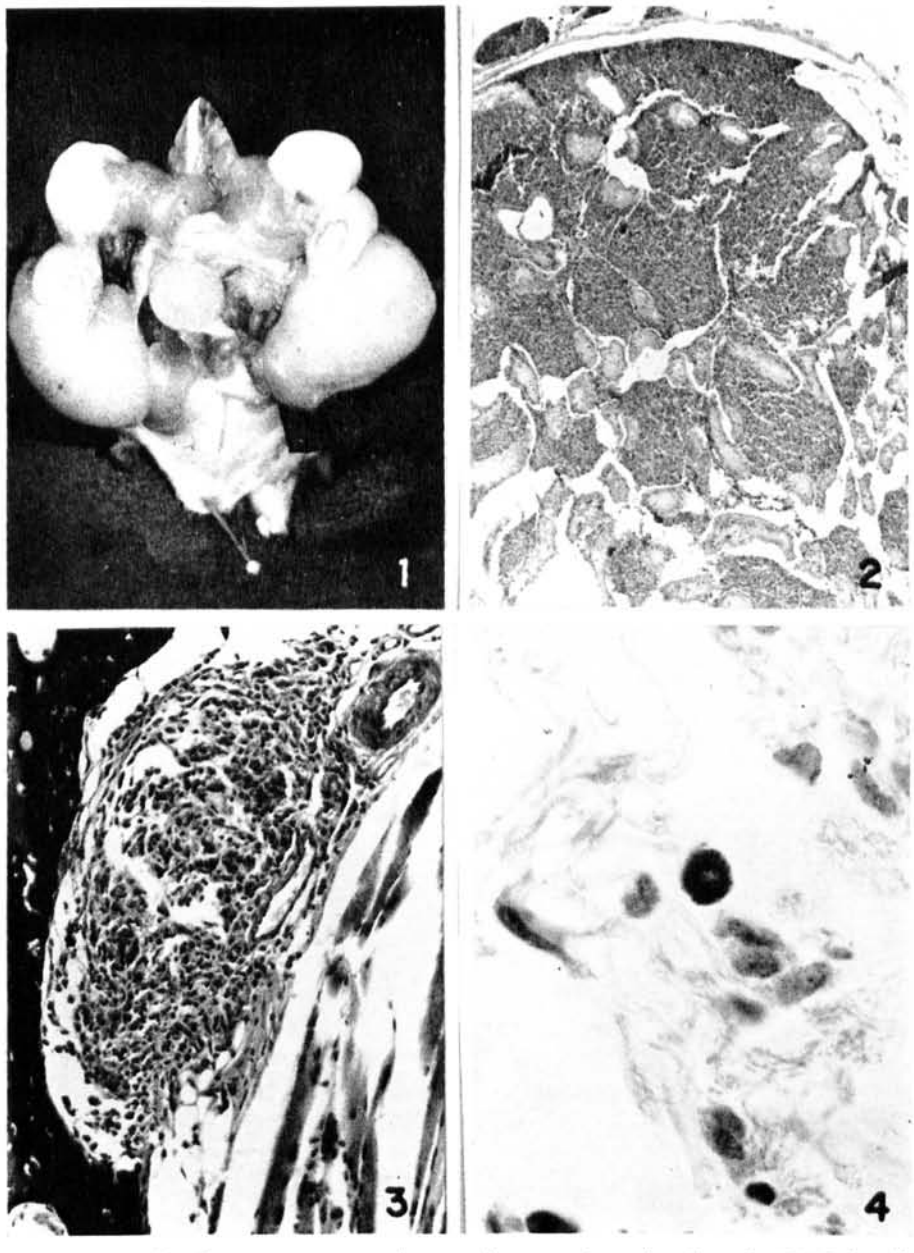


FIG. 1. Photograph of gross specimen from a hamster inoculated with ICRC bacilli. Note enlarged testis and adhesions.

FIG. 2. A section of testis of animal illustrated in Figure 1, showing partial tubular distortion and heavy histiocytic reaction in the testicular parenchyma. Fite stain, X 26.

FIG. 3. A section of foot pad showing a histiocytic granuloma. H & E stain, X 120.

FIG. 4. A section from a hamster foot pad showing fuchsinophil cell. Fite stain, X 1420.

in the foot pad lesion appeared significant.

Hamsters pretreated with silica. There was apparently no effect of silica on the rate of development of the lesion nor the severity of reaction. Out of the three animals injected, two were killed after six months and the third after 15 months. No significant differences were noted in the histopathologic reaction to bacillary inoculation in these animals (Table 1).

Experiment II. Intratesticular Inoculation in Mice

The experiment included two strains of mice, viz., dba(Bar) and hybrid mice (XVII × C57 black).

General macroscopic observations. These revealed the following changes. Limb deformities: With the exception of a few animals that died as a result of shock following operation, the mice of each strain did not show any external sign of disease for six months. Later on, although the mice were active, a few developed deformity of the forelimbs, starting with twisting and turning of the limb (Fig. 5). This twisting later progressed, with in-turning of digits, and formation of a clubfoot (Fig. 6). Ultimate loss of the forelimb was observed in three out of six affected mice (Fig. 7). These animals dragged their limbs in a way that suggested loss of muscular control of the digits. Six out of 22 dba mice (27%) exhibited these deformities.

Although dragging of a limb was observed occasionally, progressive limb deformities were not observed in the hybrid mice given intratesticular inoculations of ICRC bacilli.

Autopsy revealed that in the majority of animals in this group visceral organs other than the reproductive organs were free from macroscopic lesions. In many cases extensive adhesions of the reproductive organs were observed, so that testis, epididymis, and fat pads fused to form a hard mass. These animals possessed only rudimentary seminal vesicles and accessory glands. In some the epididymis was involved while the testis was reduced in size but appeared normal. Otherwise large nodular masses attached to the scrotal skin were found in a few animals. Occasional nodules of macroscopic size were observed in the peritoneal cavity.

Macroscopic observations. Data on individual mice are tabulated in Tables 2 and 3. Animals with postmortem changes in the tissues have been excluded from the tables. No significant difference has been noticed in the reaction of the two strains of mice to bacillary inoculation.

Testis and epididymis. As revealed in the tabulation, in a large majority of animals, lesions affected the testis and the epididymis, at times completely replacing the organs. In a few animals, the testis showed varying degrees of damage, with degeneration of the seminiferous tubules resulting in complete disruption of the testicular architecture. Histiocytic granulomas, with intracellular and extracellular acid-fast microorganisms, were common in the testicular parenchyma. In some animals there was complete distortion of the testicular tubules and replacement by the granuloma (Fig. 8). Intratubular lesions with many intracellular organisms were common (Fig. 9). Globus-like formations characteristic of lepra bacilli could be discerned clearly in the cytoplasm of cells of the tubules (Fig 10). Such lesions were closely comparable to the lesions described by Binford [3] with *M. leprae*.

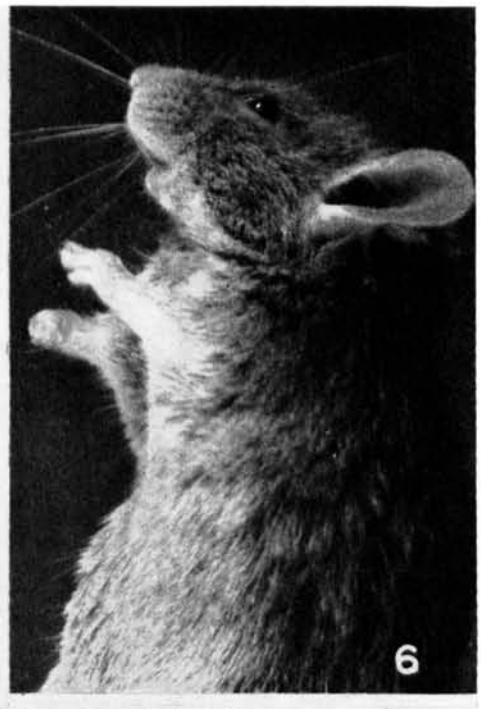
Visceral lesions. Visceral lesions were less conspicuous in the mice than in the hamsters. Only the liver showed some consistent response in both groups of mice. Small intralobular and periportal granulomata, with histiocytes containing acid-fast bacilli, were present in many mice. The spleen showed formation of giant cells, but did not contain any acid-fast bacilli. Occasionally lymph node involvement was observed. One well developed granuloma with a few acid-fast organisms was observed in the pancreas of a dba mouse.

Histology of deformed limbs. The intact and deformed limbs of the six affected animals, fixed for the silver impregnation of nerves, showed significant differences. Sections of the affected limb presented typical signs of nerve degeneration. There was marked proliferation of endoneurial and Schwann cell nuclei, and swollen, irregular and fragmented fibers were present, which

appeared tortuous, knobby and wavy, as contrasted with the smooth fibers in a section of the normal limb (Fig. 11).

DISCUSSION

In an attempt to isolate and cultivate the causative organism of human leprosy *in vitro* and transmit it to laboratory mammals, a series of experiments have been carried out in logical sequence. Details of most of these *in vitro* and *in vivo* experiments have been published ([1, 2, 6, 9]) and discussed and reviewed carefully ([8]). The present set of experiments were undertaken in order to investigate the suitability of the testicular site for establishing infection of the long cultivated ICRC bacillus originally isolated from a case of lepromatous lep-



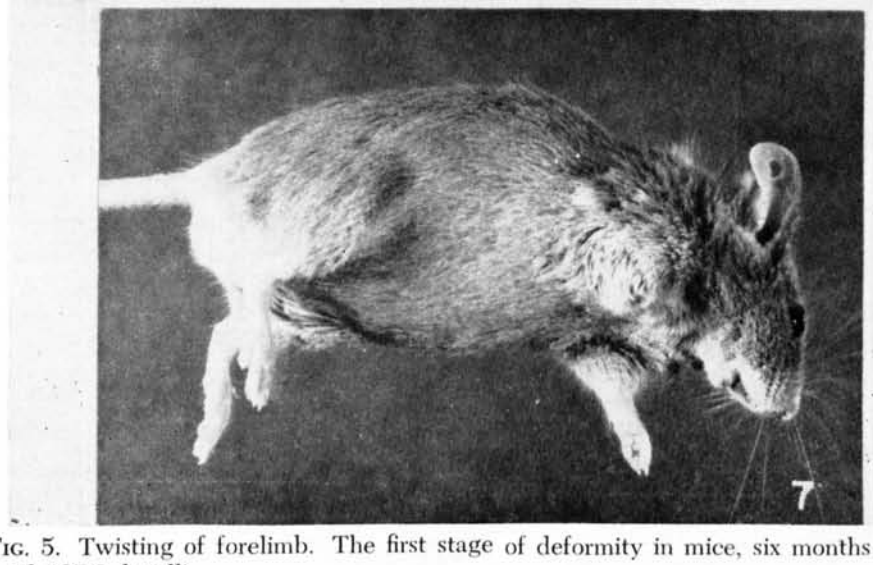


FIG. 5. Twisting of forelimb. The first stage of deformity in mice, six months after inoculation of ICRC bacilli.

FIG. 6. Development of clubfoot in a dba mouse after intratesticular inoculation of ICRC bacilli.

FIG. 7. Complete loss of limb in a mouse inoculated with ICRC bacilli.

TABLE 2. *Intratesticular inoculation of ICRC bacilli in dba mice.*

Animal No.	Postinoculation period in months	Qualitative index of histopathologic lesions			
		Testis	Epididymis	Liver	Deformity of limbs
P/2967	6	2+	—	+	Loss of limb
P/261	8	2+	—	+	Loss of limb
P/2397	9	3+	3+	+	Deformity
P/2116[a]	9	+	2+	+	Deformity
0/2025	9	3+	3+	—	Loss of limb
P/1025	9	3+	+	—	—
T/1446[a]	9	F 3+	3+	+	—
T/1445[a]	10	F 3+	3+	—	—
T/1444[a]	10	3+	3+	+	—
P/1009[a]	11	F 3+	3+	+	—
0/2803	14	2+	—	—	Loss of fingers
P/268	15	3+	3+	+	—
P/259	15	F 3+	3+	+	—
P/2966	16	F 3+	3+	+	—
P/260	16	3+	3+	+	—
P/1022	16	3+	3+	+	—
P/1595	16	F 3+	3+	+	—
P/1596	16	F 3+	3+	+	—
P/2254	16	2+	2+	+	—
0/2257	16	—	2+	+	—
0/2255	16	2+	2+	+	—
0/2024	18	2+	—	+	—
Total 22		7 21	18	18	6
Percentage		31 95	18	81	27

Note: Dose: 1×10^8 bacilli per testis. Age of animals: 3 to 12 weeks.
[a] Injected with silica prior to inoculation of bacilli.
F Fusion of testis.
+ Indicates presence of acid-fast bacilli and/or focal granuloma.
2+ Granulomata with acid-fast bacilli.
3+ Large leproma-like nodules with acid-fast bacilli and disruption of histologic pattern of the organ to varying degree.

rosy. The common occurrence of intratesticular lesions is well known in human leprosy. The lower temperature in the testicles has been considered to be a favorable factor for maintenance and growth of *M. leprae*. It was with this idea that Binford ([3]) first investigated the testicular site for establishing fresh *M. leprae* infection. He reported encouraging results. The present experiments were undertaken to repeat Binford's studies with the long cultivated acid-fast ICRC bacillus in place of fresh *M. leprae*.

Progressive histiocytic granulomas in the testicular parenchyma, at times extending into the seminiferous tubules and disrupting their structure partially or totally, were common findings in hamsters and mice inoculated intratesticularly with ICRC bacilli. Many of the lesions appeared closely comparable to those obtained by Binford with fresh *M. leprae*. The lesions were progressive, with evidence of multiplication and growth of bacilli in the cells of the tubules. Globus formation characteristic of *M. leprae* was observed in cells of the testicular tubules. The testicular site thus appeared suitable for establishing ICRC bacillary infection.

Although spread of infection to visceral organs was moderate, it was interesting to note swollen foot pads in two of the hamsters showing characteristic granulomatous lesions of the skin, with typical fuchsinophil cells. Lesions in the foot pads indicated that organ as another site suitable for in-

TABLE 3. *Intratesticular inoculation of ICRC bacilli in hybrid mice (XVII × C57 Black).*

Animal No.	Postinoculation period in months	Qualitative index of histopathologic lesions			
		Testis	Epididymis	Spleen	Liver
V/1690	3	3+	3+	+	2+
V/1726	10	F 3+	2+	±	2+
V/1920	10	F 3+	2+	+	2+
T/1124	14	—	—	—	+
T/1125	15	3+	2+	+	+
T/1126	15	3+	2+	+	+
T/1127	15	2+	2+	+	2+
T/1128	15	2+	2+	+	+
T/1459	15	3+	3+	—	+
T/881	15	3+	2+	+	+
V/1687	18	3+	3+	+	2+
V/1689	18	3+	2+	+	2+
T/883	20	3+	2+	—	+
T/884	20	3+	2+	+	+
T/885	20	3+	3+	+	+
T/1129	20	3+	3+	+	+
Total 16		2 15	15	13	16
Percentage		12 93	93	81	100

Note: Dose: 1×10^8 bacilli per testis. Age of mice: 4 to 11 weeks.
F Fusion of testis.
+ Indicates presence of acid-fast bacilli and/or focal granuloma.
2+ Granulomata with acid-fast bacilli.
3+ Large leproma-like nodules with acid-fast bacilli and disruption of histologic pattern of the organ to varying degree.

duction of ICRC bacillary infection. It appears worthwhile to plan a careful set of experiments to investigate the suitability of mouse foot pads as a site for ICRC bacillary infection on the same lines as Shepard's investigations with fresh *M. leprae*.

Besides testicular lesions the most remarkable observation in this series of experiments was the development of limb deformities. Such deformities of forelimbs, starting with twisting of limb joints, resorption of digits, and formation of clubfoot, ultimately resulting in the total loss of limb, have not been observed in the previous series of intraperitoneal inoculations of bacilli ([9]). Degeneration of nerves accompanying these deformities is a significant point. Repeated production of this condition, with further investigations on such deformities, might help to confirm the identity of the acid-fast organism maintained under culture conditions.

Induction of these deformities in mice, comparable to characteristic clinical lesions of leprosy, appears significant and adds considerably to the value of the experiments here reported. Further studies are in progress to investigate the neurotropic property of the ICRC bacillus, its immunology in comparison with *M. leprae*, and its metabolic characters. It is equally important to isolate a fresh strain of the bacillus from newer cases and study its behavior with reference to the Schwann cell in culture. It will be necessary to concentrate attention on intracellular behavior of the organism and see if adaptational (perhaps mutational) changes can be avoided if the organism is maintained inside the cell. Such investigations would give further insight into the problem of *in vitro* cultivation of *M. leprae*.

SUMMARY

This paper reports a series of experiments carried out to study the pathogenicity of the ICRC bacillus (a strain of mycobacterium isolated from human lepromatous leprosy) on intratesticular inoculation in mice and hamsters. Twenty hamsters and

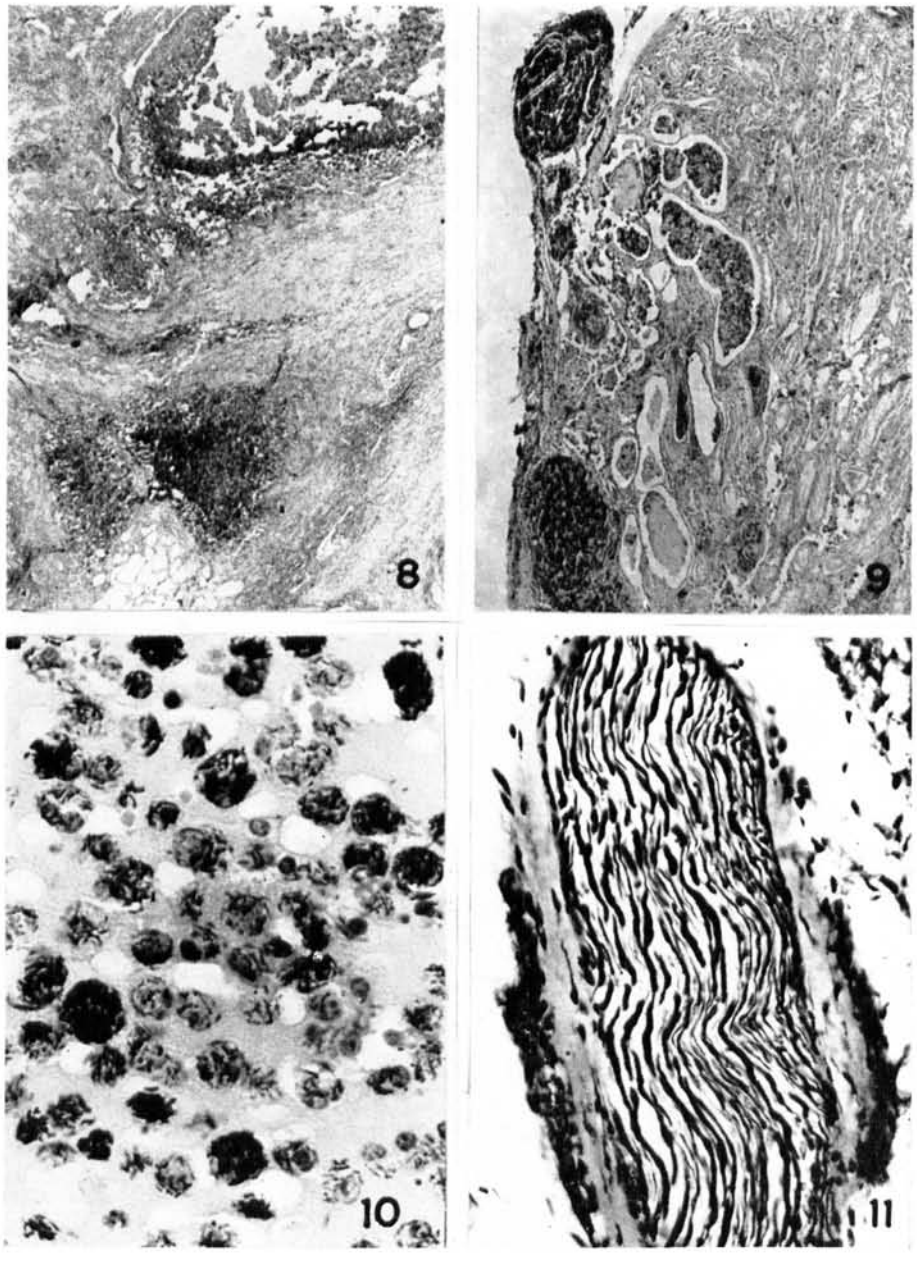


FIG. 8. A section of testis showing complete tubular distortion and replacement by a granuloma. Dba(Bar) mouse inoculated with ICRC bacilli. Fite stain, X 26.

FIG. 9. A section of mouse testis showing partial distortion of tubules, histiocytic granulomata in the parenchyma, and intratubular infiltration by acid-fast organisms. Fite stain, X 26.

FIG. 10. A section of the testis illustrated in Figure 9, under high magnification, showing intracellular proliferation of bacilli. Fite stain, X 480.

FIG. 11. A section of nerve in the deformed limb showing endoneurial nuclei and irregular fragmented fibers, indicating nerve degeneration. Silver impregnation, X 176.

42 mice of an inbred strain dba(Bar) and a hybrid strain (XVII × C57 Black) were used. Approximately 1×10^8 bacilli, maintained on the tissue culture-conditioned medium used in the experiment, were injected into the testes. Palpable lesions developed at the site in six months. Testicular granulomas were full of acid-fast bacilli, forming globus-like clusters. The testicular architecture was completely disrupted by progressive growth of the lesions. Histiocytic granulomata were found also in the liver and other visceral organs, indicating moderate spread of the bacilli. Besides these lesions at the site of inoculation, mice developed limb deformities, such as twisting of joints and resorption of digits, resulting finally in the complete loss of limb. Similarity of these lesions to characteristic clinical lesions in human leprosy appeared significant. The data presented are discussed in the light of the relevant literature.

RESUMEN

Este trabajo comúnica una serie de experimentos realizados con el fin de estudier la patogenecidad del bacilo ICRC (una raza de microbactérias aisladas de la lepra lepromatosa humana) en la inoculación intratesticular en ratones y hamsters. Fueron usados 20 hamsters y 42 ratones de una raza innata dba(Bar) y de una raza hibrida (XVII × C57 Black). Fueron inyectados en los testículos aproximadamente 1×10^8 bacilos mantenidos en un medio de condiciones de cultivos de tejidos usados en el experimento. A los seis meses se desarrollaron lesiones palpables en el sitio. Los granulomas testiculares estaban llenos de bacilos acido-alcohol-resistentes, formando pelotones globoides. La arquitectura testicular estaba completamente alterado por el crecimiento progresivo de las lesiones. También se encontraron en el higado y otros organos viscerales, granulomas histiocitarios indicando asi una moderada diseminación del bacilo. A más de estas lesiones en el lugar de la inoculación, los ratones desarrollaron deformidades de las extremidades, tales como torceduras de las articulaciones y reabsorción de los dígitos, resultando finalmente en la pérdida completa de la extremidad. Parecen significativas las similaridades de estas lesiones con las lesiones características clínicas de la lepra humana. Los datos presentados se discuten en relación con la literatura pertinente.

RÉSUMÉ

Cet article relate une série d'expériences menées afin dètudier la capacité pathogénique du bacille ICRC (qui est une souche de mycobactéries isolées de cas de lèpre lépromateuse humaine) lors de l'inoculation intra-testiculaire chez la souris et le hamster. Vingt hamsters et 42 souris, appartenant soit à une souche consanguine dba(Bar), soit à une souche hybride (XVII × C57 Black), one été utilisés. On a injecté dans les testicules environ 1×10^8 bacilles provenant du milieu adapté pour cultures de tissu employé au cours de ces expériences. Des lésions palpables se sont développées à l'endroit d'inoculation endéans les six mois. Les granulomes testiculaires étaient remplis de bacilles acido-résistants, qui formaient des agrégats ayant les apparences de globi. La structure testiculaire a été complétement bouleversée par suite de l'extension progressive des lésions. Des granulomes histiocytaires ont également été découverts au niveau du foie et d'autres organes internes, ce qui témoigne d'une dissémination modérée des bacilles. Outre ces lésions notées à l'endroit d'inoculation, les souris ont développé des déformations des membres, telles que foulure des articulations et résorption des doigts, avec comme résultat ultime la perte complète du membre. Il semble y avoir une nette resemblance entre ces lésions et les lésions cliniques caracteristiques de la lèpre humaine. Les résultats présentés ici sont discutés à la lumière des données pertinentes de la littérature.

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