EXPERIMENTAL INFECTION OF THE GOLDEN HAMSTER WITH *MYCOBACTERIUM LEPRAE*[1]

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Adler (1) was the first to attempt the transmission of human leprosy to the golden hamster (*Cricetus auratus*). He claimed that bacterial multiplication occurred within 6 weeks of implanting lepromatous tissue. Burnet (8, 9), and Burnet and Jadfard (10) also reported successful transmission. In contrast, however, only very cautious claims were made by Doull and Megrail (16); Carpenter and Naylor-Foote (11) have been unable to repeat their initial success; and negative results have been reported by Dubois and Gavrilof (17), Oteiza and Blanco (27), and Marchoux *et al.* (21). Moreover, the last named authors drew attention to the ability of living and dead leprosy bacilli to remain for long periods in the animal body and argued that the finding of such bacilli several months after inoculation was not in itself evidence of progressive infection.

In 1956, Binford (4) inoculated 100 hamsters with suspensions of bacilli prepared from chilled or frozen human leprosy tissues. Because in man the leprosy bacillus appears to have a predilection for the cooler areas of the body, the hamsters were inoculated in the ears and testes. Some animals were given total body irradiation and/or cortisone in an attempt to lower resistance to experimental infection. Histiocytic granulomata were found in 22 of the 85 hamsters examined; intraneural acid-fast bacilli were present in lesions in 3 ears. Control animals given heat-killed material did not develop lesions. Hamster to hamster passage was successful, but it was subsequently discovered that the lesions were due to a cultivable mycobacterium (5, 6), considered by Shepard to belong to the group of nonphotochromogens (3). Further human to hamster experiments have been commenced by Binford (5, 6) and the finding of microscopic lesions containing many well stained, noncultivable, intracellular, and sometimes intraneural, acid-fast bacilli in a number of animals has been reported briefly (7).

Following Binford's earlier claims, Convit and his colleagues (13, 14) inoculated approximately 600 hamsters with fresh suspensions prepared from biopsies from patients suffering from different types of leprosy. Initially, of 26 strains used, 2 only, both derived from "borderline" patients, produced localized lesions in hamster ears; these contained large numbers of acid-fast bacilli. Each strain has been passaged serially, with increasingly shorter incubation periods, to

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ears, testes, foot pads and cheek pouches. Neither strain has been isolated on Loewenstein-Jensen medium, although, more recently, a mycobacterium has been cultivated from lesions in hamsters that had been inoculated with a suspension derived from human lepromatous tissue ([15]). On the other hand, Shepard ([29, 31]), who also inoculated a similar number of hamsters in the ears and testes, was able to identify acid-fast bacilli in very few animals and then only in small numbers.

In an attempt to confirm Binford's earliest report ([3]), and on the advice of R. J. W. Rees, a series of experiments was started in 1959 using the golden hamster; these have now reached the fifth passage. We present here a detailed account of the human to hamster and the first passage hamster to hamster experiments.[3]

MATERIALS AND METHODS

I. HUMAN TO HAMSTER EXPERIMENTS

Source and preparation of inoculum.—Ear trimmings or skin biopsies were removed on separate occasions from 3 untreated lepromatous or borderline lepromatous patients (A, B and C) and were immediately processed. The epidermis of each specimen was removed, and the tissue was cut finely with scissors, ground, suspended in sterile normal sulfae, and centrifuged at 1,000 r.p.m. for 5 minutes. The supernatant below the lipid layer was removed, and stored at 4°C until a bacillary count for *M. leprae* had been performed. The suspensions were inoculated into hamsters within 5-24 hours after taking the lepromatous tissues.

Counts of the suspensions were made by a modification of the method of Hart and Rees ([18]), in which the bacilli, including those in groups, were counted individually. The results were as follows:

Suspension A—2.1×10^7 bacilli/ml.
Suspension B—4.1×10^8 bacilli/ml.
Suspension C—6.9×10^7 bacilli/ml.

The morphology of suspension A was not noted. Suspension B contained 42 per cent normal and 58 per cent degenerate organisms as judged by electron microscopy ([22]). Suspension C could not be freed sufficiently of tissue debris for electron microscopy, although a smear showed that 51 per cent of bacilli were solid-staining. Aliquots from each of the three suspensions were heated in a water bath at 70°C for an hour to supply heat-killed control inocula.

Inoculation.—Fifty-three male hamsters, 4-5 months old, weighing 80-140 gm., were inoculated in three batches, 10 receiving suspension A, 14 suspension B, and 29 suspension C. Twenty-one animals received approximately 300 R whole body irradiation before inoculation, but, as there is no evidence that their resistance to *M. leprae* was altered, no separate analysis will be presented.

The animals were anesthetized with sodium pentobarbitone given intraperitoneally. Each hamster acted as its own control, living suspension being inoculated into the left ear and left testis, and equal volumes of the corresponding heat-killed suspension into the right ear and right testis. Approximately 0.1 ml. of suspension was inoculated between the epidermis and the cartilage of the upper surface of each ear; a conspicuous localized bleb was always produced, which soon disappeared. A dose of 0.2 ml. was injected into the substance of each testis. The dosage for each site was therefore in the range of 10^6–10^7 bacilli. Tuberculin syringes with 1 cm., 27 gauge platinum needles were used, the needle being flamed between inoculations. Thereafter the hamsters were kept in a nonairconditioned animal house with average day and night temperatures of 32° and 24°C.

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[3]A preliminary communication was given at the VIIIth International Congress of Leprology, Rio de Janeiro, September 1963.

Examination.—Both testes were examined bacteriologically or histologically; both ears from 23 animals were examined histologically.

(a) *Bacteriologic,*—(1) Left testis: Each left testis was removed aseptically and placed in a glass homogenizer. One per cent albumin saline was added, 1 ml. to testes weighing less than 0.5 gm., and 1.5 ml. to those over 0.5 gm. The testis was homogenized and centrifuged, and the supernatant removed and kept at 4°C. A Ziehl-Neelsen-stained smear of the supernatant was examined, and from an assay of 100 oil immersion fields, it was estimated that yields of approximately 1×10^4 bacilli were detected. Four of the positive suspensions were also successfully counted. (2) Right testis: An impression smear was made as a routine. In some cases suspensions were also prepared and smears examined.

(b) *Histologic.*—Tissues were fixed for 6 hours in a freshly prepared mixture of saturated mercuric chloride (96 ml.) and glacial acetic acid (4 ml.), and then transferred to 70 per cent alcohol. The ears were cut on a line parallel with the long axis into 3-5 narrow slices, depending on the size, and the testes were cut into 2 or 3 pieces. After embedding in paraffin wax, the specimens were cut in sections at 4μ, adjacent sections being stained by the Ziehl-Neelsen method, hematoxylin and eosin, and the periodic acid-Schiff (PAS) procedure. Turnbull's method for iron-containing pigment also was used occasionally. In all instances in which no mycobacteria were detected in specimens inoculated with viable mycobacteria, further sections were examined and a similar search was carried out if any unusual histologic findings, such as focal accumulations of mononuclear cells, were observed in specimens inoculated with inactivated mycobacteria.

II. FIRST PASSAGE, HAMSTER TO HAMSTER EXPERIMENTS

Source and preparation of inoculum.—Left testicular suspensions prepared from 9 primary inoculation hamsters, all of which contained solid-staining mycobacteria, were used. Details of the suspensions are given in Table 1. No acid-fast organisms were isolated on Loewenstein-Jensen medium from the suspension used for animal passage to inoculate hamsters P2-P5 and P12-P19. Facilities for culturing the suspensions used for the inoculation of hamsters P6-P11 were not available.

Inoculation.—The testicular suspensions listed in Table 1 were used to inoculate 18 hamsters, 6 weeks to 4 months old. No animal was irradiated. As the volume of testicular suspension was in every case very small (<1.5 ml.) no heat-killed controls were used. Testes and ears were again chosen as inoculation sites, the ears receiving 0.1 ml. of suspension, and testes 0.1 or 0.2 ml. One animal was inoculated in both testes, and a second (No. P11) in both testes and one ear; all other first passage hamsters were inoculated in one ear and one testis.

TABLE 1.—*Details of suspensions obtained from the left testes of hamsters killed 19-22 months after inoculation with suspensions of* Mycobacterium leprae *prepared from human tissue.*

Primary inoculation hamster number	Inoculum given (No. of bacilli)	Bacillary yield	Percentage of solid-staining bacilli	First passage hamster no. inoculated with suspension
2	4.2×10^6	N.C.[a]	20	P3
5	4.2×10^6	N.C.[a]	(2/4)	P2
16	8.3×10^7	N.C.[a]	(2/2)	P4
20	8.3×10^7	N.C.[a]	71	P5
29	1.4×10^7	5.4×10^4	(1/2)	P8,P9,P10
30	1.4×10^7	3.0×10^6	18	P12,P13,P14,P15
32	1.4×10^7	3.1×10^6	27	P6,P7
45	1.4×10^7	3.0×10^6	18	P16,P17,P18,P19
53	1.4×10^7	N.C.[a]	6	P11

[a]N.C.—No count made.

Examination.—The animals were killed after 17-19 months, except for one hamster that died at 15 months. The inoculation sites from 9 hamsters were examined histologically, as in the human to hamster experiment. Ears and testes from the other 9 animals were sent on wet ice by air from the Sungei Buloh Leprosarium to the National Institute for Medical Research. Suspensions were prepared from the testes by essentially the same technic as before. Each ear was shaved with a razor blade, cut finely with scissors and ground with sterile sand and a pestle in a mortar. A measured volume (usually 4 ml.) of 1 per cent albumin saline was added slowly to make a crude suspension, which was then centrifuged, and the resulting supernatant was examined in smear preparation and counted for acid-fast bacilli.

RESULTS

I. PRIMARY INOCULATION—HUMAN TO HAMSTER

Ears.—Macroscopic examination: Clinical examination of the ears was negative; no area of thickening, nodulation or alteration of pigment was detected. As it was planned to attempt passage only from ears showing macroscopic abnormality, no suspensions were prepared, and no bacterial counts were made.

Histologic examination: The hamster ear is covered by thin squamous epithelium, 3-4 cells thick, the stratum lucidum and stratum granulosum being poorly represented and melanin pigment being absent in the basal layer. Hair follicles, more numerous on the dorsal than on the ventral aspect, are fairly uniformly distributed, and sebaceous glands open into the lumen of the follicle about half way from its base to its external aperture; sweat glands are present only at the base of the ear. The cartilage is of hyaline type and is defined by a moderately dense perichondrium containing elongated cells. The continuity of the cartilage is interrupted occasionally by thin septa of connective tissue. Lying parallel and close to the cartilage on its ventral aspect are thin bundles of skeletal muscle; on the dorsal aspect, skeletal muscle is found close to the ear attachment. The remainder of the ear consists of connective tissue in which lie blood vessels, lymphatic channels and nerves. Melanin-containing cells are a conspicuous feature of the connective tissue, and are more numerous on the dorsal than the ventral aspect. They have a striking dendritic appearance, which is due to their elongated branching processes. It has not been possible to determine whether they are chromatophores (cells that have phagocytosed melanin produced by melanoblasts) or are true melanoblasts. In this communication, the term melanophore is used. Tissue mast cells also are occasionally found.

Twenty-three pairs of ears from inoculated hamsters were examined at periods of 5 to 22 months. In hamster 49, killed 15 months after inoculation, the ears were ulcerated and showed extensive subacute inflammatory change. No mycobacteria were found and this animal is omitted from the analysis. In hamster 40, killed 22 months after inoculation, both ears showed an unusual pattern of histologic change and widespread distribution of acid-fast bacteria; it will be described separately.

In the remaining 21 animals, intracellular acid-fast bacteria, morphologically identical with *M. leprae*, were found in the ears inoculated with viable organisms in 16 animals. In hamster 41, killed 5 months after inoculation, 3 mononuclear cells were found in the right ear, inoculated with inactivated mycobacteria, each containing one well-stained acid-fast organism; none was found in the left ear, which had been infected with viable mycobacteria, and considerable search of the right ear failed to reveal any more organisms. In hamster 23, killed after 22 months, mycobacteria were readily found in the left ear, and in the right ear a small group of cells within a nerve was found to contain solid-staining mycobacteria. No additional foci of mycobacteria-containing cells were found in any of the additional sections examined. An analogous situation was found in hamster 45. In the right ear, 3 mycobacteria were found in one mononuclear cell. Extensive search of further sections failed to reveal any more infected cells. As in hamster 23, the left ear, inoculated with viable microorganisms, showed typical acid-fast organisms in cells of various kinds (Table 2). These were the only instances in which acid-fast organisms morphologically resembling *M. leprae* were detected in sites injected with inactivated microorganisms.

As already stated, in the 21 hamster ears about to be described, no

TABLE 2.—*Distribution of acid-fast organisms in ears inoculated with viable* M. leprae.

Hamster number	Ep	HF	Mel	M	Mu	N	P	C	E	T
1	+	+	+	+	+	+	+	−	−	14
3	+	+	−	+	+	+	+	−	−	10
4	−	−	−	+	−	−	+	−	−	12
9	−	−	−	+	−	−	+	−	−	12
10	−	−	−	−	−	−	−	−	−	10
23[a]	−	−	−	+	−	+	+	−	−	22
27	−	−	−	−	−	+	−	−	−	20
29	−	−	−	−	−	+	+	−	−	21
30	−	−	+	−	−	+	+	−	−	22
32	−	−	−	+	−	+	−	−	−	22
35	−	−	−	−	−	−	−	−	−	22
41[a]	−	−	−	−	−	−	−	−	−	5
42	−	−	−	−	−	+	+	−	−	5
43	−	−	−	+	−	+	+	−	+	22
45[a]	−	−	−	+	+	+	+	−	+	22
46	−	−	−	−	−	+	+	−	−	22
47	−	−	−	−	−	−	−	−	−	22
48	−	−	+	+	+	+	+	+	−	22
52	−	−	−	−	−	+	+	+	−	21
53	−	−	−	−	−	−	+	−	−	22
54	−	−	−	−	−	−	−	−	−	22

Ep = Epidermis; **HF** = Hair follicle; **Mel** = Melanophores; **M** = Mononuclear cells **Mu** = Muscle; **N** = Nerve; **P** = Perichondrium; **C** = Cartilage; **E** = Endothelium; **T** = Time in months after inoculation.

[a]Mycobacteria found in right ear. See text.

macroscopic changes were observed and gross microscopic lesions were absent. As compared with the ears inoculated with inactivated mycobacteria, however, there was a general increase in cellularity, involving especially the fibrocytes and pigment-containing cells, and occasionally the squamous epithelium was hyperplastic. Focal accumulations of mononuclear cells between epidermis and cartilage were seen fairly frequently, and occasionally there was an increased cellularity within a nerve. A slight degree of fibrosis of skeletal muscle fibers close to the perichondrium was sometimes seen and occasionally slight irregularity of the cartilage rods. Lymphocytic or plasma cell infiltration was never found.

In 16 left ears from hamsters injected with viable mycobacteria, intracellular acid-fast microorganisms were found in nerves, perichondrial cells, cartilage cells, skeletal muscle, endothelial cells, squamous epithelium and hair follicle cells, and melanophores. It must be emphasized, however, that in all instances, the distribution of the mycobacteria was focal and irregular. Frequently, further exploration of a positive sample failed to reveal more infected cells, and, conversely, a search in other areas sometimes disclosed intracellular mycobacteria in a specimen considered initially to be negative. The results are summarized in Table 2.

Nerves.—In general, it was possible to be certain that the mycobacteria were intracellular. They were sometimes found in cells of the epineurium but more frequently in cells in close relation to the axons. By the methods used it was not possible to differentiate between Schwann cells and endoneurial cells. No evidence was found to suggest that the microorganisms were present within the axons (Figs. 1 and 2).

Perichondrium.—This was an equally common site and easy to examine at high magnifications. Thin elongated cells, lying parallel with and close to the cartilage rods, were often stuffed with mycobacteria; they were most numerous on the inner aspect of the perichondrial membrane (Fig. 10).

Cartilage.—In only 2 instances were mycobacteria found in cartilage cells (Figs. 8 and 9). In both specimens, the infected chondrocytes were smaller than usual and it seems possible either that the cells had become parasitized at a stage when they were becoming

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FIG. 1. Segment of a nerve showing intracytoplasmic mycobacteria. Hamster 1. X1600.

FIG. 2. Oblique section of a nerve. Mycobacteria are present also in perineurial cells. Hamster 3. X2400.

FIG. 3. Cross section of a skeletal muscle fiber. Two mycobacteria occur within the muscle substance. Hamster 48. X2600.

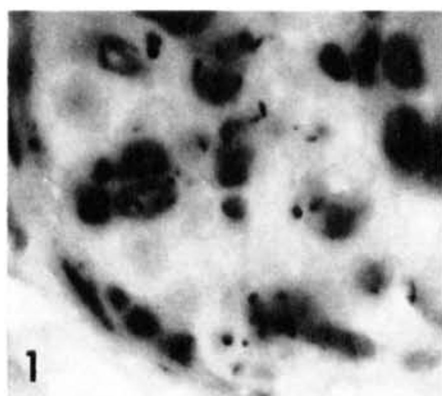
FIG. 4. Oblique section of skeletal muscle fiber showing clumps of mycobacteria within the muscle substance. Myofibrils are visible. Hamster 48. X2400.

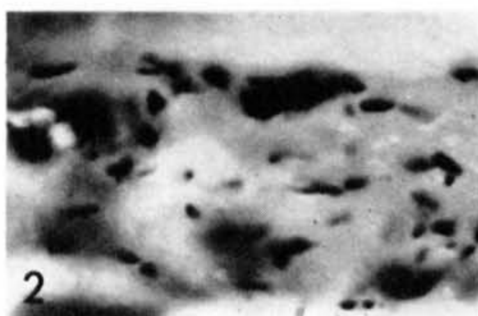
FIG. 5. Mycobacteria are present in sarcolemma cells closely applied to a muscle fiber Hamster 48. X1700.

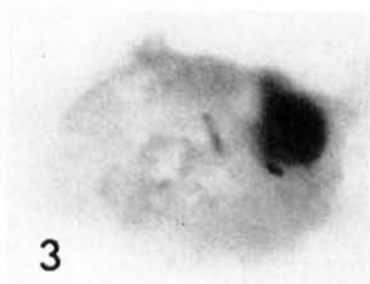
FIG. 6. Numerous mycobacteria are present within a skeletal muscle fiber cut in longitudinal section. Hamster 48. X1700.

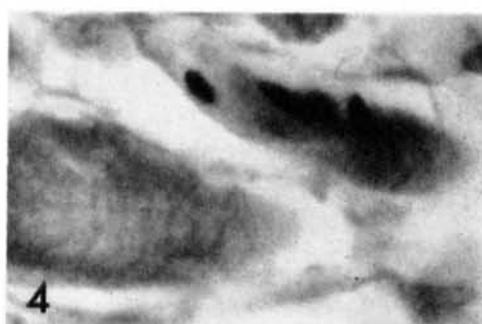
All the sections were stained by the Ziehl-Neelsen method and counterstained with hematoxylin.

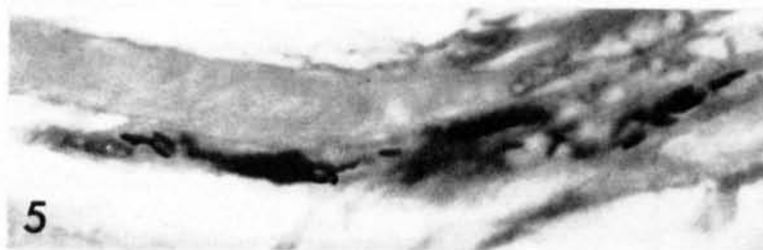
transformed from chondroblasts into chondrocytes or that during repair following damage at inoculation, or incidental damage during the observation period, infected perichondrial cells had become transformed into chondrocytes. With this exception, no abnormality was found in the cartilage, even in specimens in which fairly numerous perichondrial cells were found to be infected.











Skeletal muscle.—In the 4 animals in which mycobacteria were found in skeletal muscle, microorganisms were found also within cells in nerves. Where the muscle fibers were cut in cross-section, the mycobacteria occurred either singly or in closely packed groups, sometimes within a vacuole of the muscle substance or in close relation to a muscle cell nucleus (Fig. 3). In longitudinal or oblique section, they appeared to lie irregularly parallel to and within the muscle fiber (Figs. 4, 6 and 7). Mycobacteria were found also in sarcolemma cells closely applied to the basement membrane of the muscle fiber (Fig. 5). Recent electron microscopy of cardiac muscle by Lannigan ([20]) has shown that cells closely applied to the sarcolemma have processes that indent the muscle substance. A similar pattern may exist in skeletal muscle, and microorganisms apparently within the muscle fiber may in fact be lying within such a cell process. Evidence of muscle damage, such as multinucleated plasmodial formation, was never found, but myofibrils were less conspicuous in those containing mycobacteria. Damage to the tissues of the ear at inoculation can never be excluded and thus the possibility that the mycobacteria gained access to the muscle cells following inoculation injury and remained *in situ* while regeneration took place, must also be kept in mind. A focal increase of mononuclear cells and fibrocytes was sometimes found between individual muscle fibers.

Mononuclear cells.—It was rather surprising that the incidence of mycobacteria within individual mononuclear cells or in collections of mononuclear cells was of a lower order than in nerves, or perichondrial cells (Table 2). In no instance did the numbers present within cells approach those found in murine leprosy or in lepromatous leprosy in man (Fig. 11). Occasionally, focal collections of cells were found, of characteristic oval or elongated shape and eccentrically placed nuclei, but containing only very fine granular brownish material; sometimes these cells gave a faint diffuse reaction for iron. Such areas have always been examined with especial care, and occasionally a single solid-staining mycobacterium or one or two irregularly staining microorganisms have been found lying among the granules. Attention is drawn to this finding, which may be of significance in relation to the life history of *M. leprae* in hamster cells.

Epidermis and hair follicle epithelium.—In 2 animals (hamsters 1 and 3) isolated or small groups of mycobacteria were found in the

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FIG. 7. Oblique section of a muscle fiber showing a group of mycobacteria in a vacuole just within the sarcolemma. Hamster 48. X3000.

FIG. 8. Chondrocyte just within the perichondrium stuffed with mycobacteria. Hamster 48. X1000.

FIG. 9. Focus of small chondrocytes containing groups of mycobacteria. Hamster 52. X1000.

FIG. 10. Perichondrial cell containing clumps of mycobacteria, obscuring the nucleus. Hamster 48. X1700.

FIG. 11. Group of mononuclear cells with abundant cytoplasm, containing mycobacteria. Hamster 43. 1400.

All the sections were stained by the Ziehl-Neelsen method and counterstained with hematoxylin.

basal layer of the epidermis and in the flattened surface cells (Figs. 12 and 14). Free mycobacteria were also seen lying in the keratinous debris (Figs. 12 and 14). A few cells in the epithelium lining a hair follicle have also been seen to contain mycobacteria (Fig. 13). It is of interest that in one animal, mycobacteria were found also in the melano-

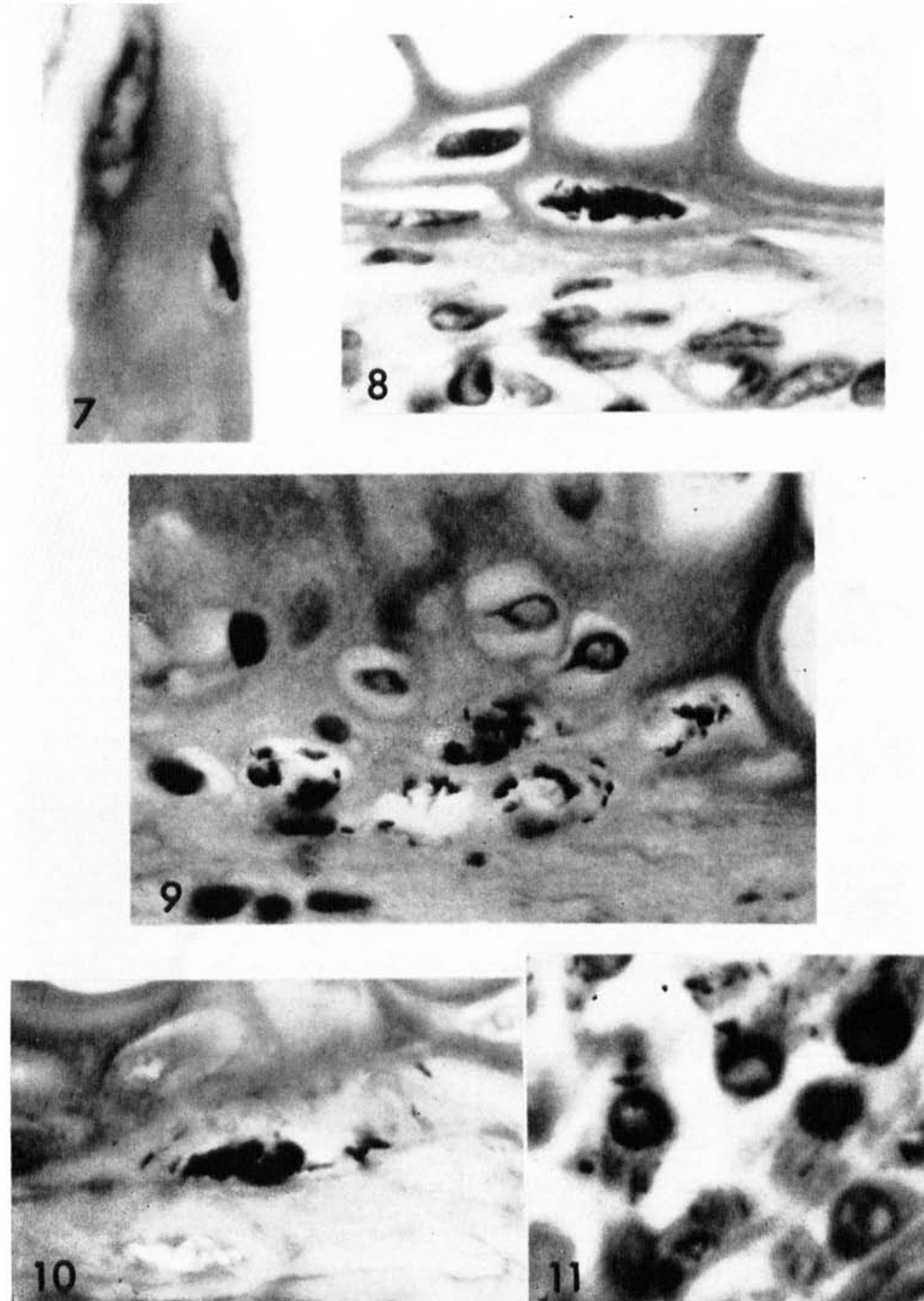


TABLE 3.—*Distribution of acid-fast organisms in ears of 1st passage hamsters.*[a]

Hamster number	Ep	HF	Mel	M	Mu	N	P	C	E	T
P2	+	+	+	+	—	—	+	—	+	18
P4	—	—	—	—	—	—	—	—	—	18
P7	+	+	+	+	+	+	+	—	+	17
P10	+	—	+	+	—	+	+	—	—	17
P13	—	—	—	—	—	—	—	—	—	18
P15	—	—	+	—	—	—	+	—	—	18
P17	+	—	—	—	—	+	+	—	—	18
P19	+	—	—	—	—	+	+	—	—	18

[a]See Table 2 for key.

phores (Figs. 12 and 13), nerves, skeletal muscle, mononuclear cells and perichondrium. Although ordinarily the epidermis is only 2-4 cells thick, hyperplastic areas sometimes occur in which the usual features of thick mammalian epidermis, stratum spinosum and stratum granulosum, are found. This change has not been found to have any relationship to the occurrence of mycobacteria in epidermal cells, and is considered to be the result of incidental trauma.

Melanophores.—Melanin-containing cells are a feature of the hamster ear and are frequently increased in number, especially on the dorsal aspect. They always maintain the typical dendritic appearance, however. In 3 specimens many were found to contain typical acid-fast organisms, often situated within the long-branching processes (Figs. 12 and 13). Sometimes long processes containing mycobacteria were seen in close relationship to the basal layer of the epidermis. In general the pigment content tended to be diminished when the microorganisms were numerous.

Vascular endothelium.—In hamsters 43 and 45, in which mycobacteria were found fairly easily in several sites, a single solid-staining microorganism was found within an endothelial cell of a vascular capillary. In 2 first passage animals a similar observation was made (Table 3).

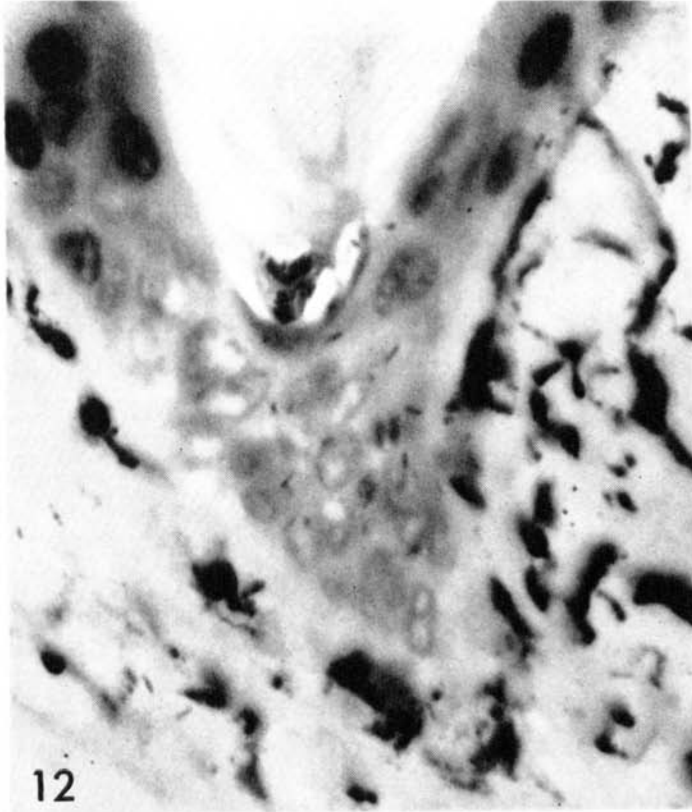
In hamster 40, the tissues of both right and left ears were indistinguishable, showing extensive infiltration with mononuclear cells and cells of epithelioid type, many of which were stuffed with acid-fast microorganisms. Organisms were abundant also in cells within nerves and in the epineurium, in muscle, perichondrium, and melanophores,

FIG. 12. Squamous epithelium and subepithelial dermis with "dendritic" melanophores, close to a hair follicle. Mycobacteria are present in the epithelial cells and a clump is visible in the keratin on the surface. Mycobacteria are also present in the dendritic processes of the melanophores. Hamster 1. X1600.

FIG. 13. Section through the base of a hair follicle unit showing mycobacteria in the epithelial lining cells. They are present also in the processes of melanophores. Hamster 1. X1600.

FIG. 14. Thin squamous epithelium with intracytoplasmic mycobacteria and mycobacteria lying free in the keratinous debris. Hamster 3. X3000.

All the sections were stained by the Ziehl-Neelsen method and counterstained with hematoxylin.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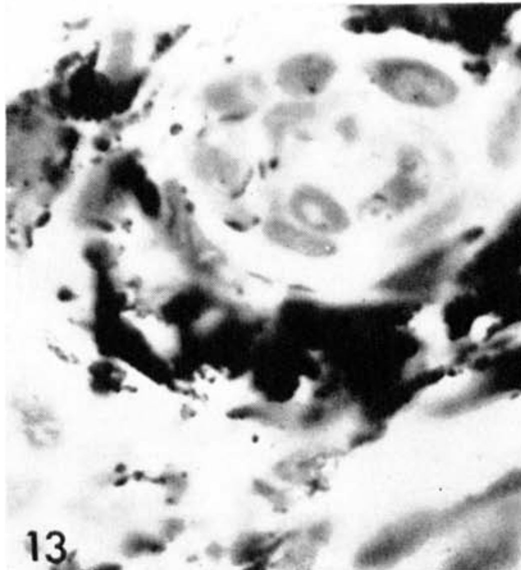
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in the walls and endothelium of blood vessels and lymphatic channels, and in epidermal and hair follicle cells. They were also found abundantly in extracellular spaces and the lumina of blood vessels, and were very numerous among desquamated epidermal cells. Morphologically the microorganisms were longer and thinner than *M. leprae* as identified in the other animals of this study. The significance of these observations will be discussed later.

Testes.—The epididymis was generally included in the samples taken for histologic survey. No unusual features are present in the testis of the hamster. The interstitial cells of Leydig appear in general to be less numerous than in the mouse or rat, and as age advances, the testes become atrophic and spermatozoa are no longer produced. At the same time, the epithelial cells and the interstitial cells come to contain varying amounts of acid-fast granular pigment, the granules varying in size from 2-3μ to just within the limits of resolution; these granules also give a PAS-positive reaction and the cells sometimes show a weak reaction for iron. This material can thus be distinguished histochemically as well as morphologically from mycobacteria in tissue sections. Furthermore, the material was equally abundant in testes inoculated with inactivated mycobacteria. In mononuclear cells in the stroma of the testes, very fine acid-fast material was sometimes observed in the material examined from animals inoculated with viable mycobacteria and to a lesser extent in animals with inactivated mycobacteria (see mononuclear cells, page 304). In only one instance, however, hamster 52, were typical mycobacteria found in the testis injected with viable organisms. These occurred in a localized collection of somewhat elongated mononuclear cells just within the capsule of the atrophied testis. They showed uniform staining, and no degenerate forms were detected. Further examination of the testis failed to reveal any other foci of acid-fast bacteria.

Bacteriologic examination.—Bacteriologic examination was made on 19 pairs of testes from animals killed 19 to 22 months after primary inoculation. All right testes were negative, except that, in one smear only, a single irregularly staining and therefore presumably dead, acid-fast bacillus was seen. Nine of the 19 left testicular suspensions contained solid as well as irregularly staining acid-fast bacilli (Table 1). Counts were made of 4 of these. The highest yield, however, was only 3.1×10^6 bacilli from a testis originally inoculated with 1.4×10^7 organisms (i.e., the yield was only 22 per cent of the original inoculum) and in every case a fall in bacillary population had occurred. Moreover, in the 3 suspensions with the highest yields, only 27, 18 and 18 per cent of the organisms were solid-staining. These results suggest that although viable-looking *M. leprae* may frequently remain for many months in hamster testes, the numbers gradually diminish. No mycobacteria were isolated from those suspensions cultured on Loewenstein-Jensen medium.

II. FIRST PASSAGE—HAMSTER TO HAMSTER

Histologic examination.—The inoculated ears and testes from each of 8 animals of the first passage series were examined histologically, the testes only from one animal. In no instance were unequivocal mycobacteria found in any of the 10 testes examined.

In contrast, however, mycobacteria were found in 6 out of the 8 hamster ears examined. Since they were observed in sites similar to those involved in the primary inoculation series, it is unnecessary to repeat the detailed description given earlier (pages 300-302). The distribution in individual hamsters is given in Table 3. The incidence in squamous epithelium was higher than in the primary series, in 5 out of the 8 ears examined, and in 2 of these (hamsters P10 and P19), very scanty extracellular mycobacteria were found among the non-nucleated superficial squames.

Bacteriologic examination.—Testes: Suspensions were prepared from the left testes of 9 animals, and the one inoculated right testis. When mycobacteria were seen, the material was cultivated on Loewenstein-Jensen medium. Eight testicular suspensions were negative. The ninth contained 4 solid and 2 irregularly staining bacilli in 100 fields; the bacilli resembled *M. leprae* morphologically, no growth was obtained on Loewenstein-Jensen medium at 37° and 34°C, and by analogy with the human to hamster experiments it is considered probable that the few bacilli harvested were slowly decreasing survivors of the inoculum. The tenth one, from the left testis of hamster P11, whose right testis also was inoculated, contained numerous acid-fast organisms. The yield was 2.3×10^7 bacilli with 63 per cent solid-staining, but the bacilli were shorter and stouter than *M. leprae;* no growth was obtained on Loewenstein-Jensen medium. Hamsters (second passage) and mice have been inoculated with the two positive testicular suspensions.

Ears: Homogenates of 9 ears from 9 animals contained acid-fast bacilli morphologically resembling *M. leprae*. Counts on 4 confirmed a ten-fold increase. However, the yield never exceeded 10^6 (Table 4). It has not proved possible to grow the mycobacteria on culture at 37° and 34°C. The 9 suspensions were used for further (second) passage to hamsters (ears and foot pads), and mouse foot pads have also been inoculated.

DISCUSSION

Currently, two main types of infection have been described following the inoculation of *M. leprae* into experimental animals. Chatterjee ([12]), Convit ([13, 14, 15]), and Bergel ([2]) reported the production of relatively massive infections, containing very numerous mycobacteria. On the other hand, Shepard ([29, 31]) has obtained only limited multiplication in the mouse foot pad, with a ceiling yield of 10^6-10^7 bacilli. His work has been confirmed by Rees ([28]) and by Janssens and Pattyn ([19]). We ourselves initially modelled our experiments on those of

TABLE 4.—*Details of suspensions obtained from ears of first passage hamsters killed 17-19 months after inoculation of suspensions of* Mycobacterium leprae *prepared from hamster testes.*

First passage hamster number	Inoculum given (No. of bacilli)	Bacillary yield	Percentage of solid-staining bacilli
P5	N.C.[a]	2.4×10^5	47
P6	3.1×10^5	4.9×10^6	56
P8	5.4×10^3	—[a]	86
P9	5.4×10^3	—[b]	(3/3)
P11	N.C.[a]	8.3×10^5	72
P12	3.0×10^5	3.8×10^6	69
P14	3.0×10^5	3.8×10^6	70
P16	3.0×10^5	—[c]	48
P18	3.0×10^5	6.0×10^6	42

[a]N.C.—No count made.
[b]Too few organisms for accurate count.
[c]Too much debris for accurate count.

Binford ([3, 4]) (see page 298-299), and used large inocula in the first instance, examining the ears regularly for macroscopic lesions; as none developed, the primary inoculation ears were examined histologically only, not bacteriologically. However, those bacterial counts that were undertaken, in both the human to hamster and the first passage animals, have given yields not exceeding 10^6 bacilli. In addition, when the inoculating dose was less than 10^6 (in the first passage), multiplication to this level was detected. Therefore, with inocula of the order of 10^6-10^7 bacilli there was no detectable multiplication, but in ears with smaller inocula, limited multiplication occurred. This limited multiplication has been amply confirmed by further passage, as will be reported; the importance of including bacterial counts in such experiments is evident. Moreover, the histologic findings, which were similar in the ears of primary inoculation and of first passage hamsters, are compatible with those of Shepard, if allowance be made for the differences in the animal species used, and the tissues examined. Therefore we conclude that the type of infection we have obtained in the hamster is analogous to that described by Shepard in the mouse.

The histologic study has revealed that a wide variety of tissues may be involved, and it is also of value, with certain reservations, in regard to the findings in human disease. Large granulomatous collections of cells packed with mycobacteria were absent, but the involvement of nerves has an analogy with human leprosy. So far as we know, the involvement of perichondrial cells in nasal leprosy in man has not been studied; and since in our material acid-fast bacilli have been very frequently found in perichondrium, it would be of interest to examine nasal cartilaginous biopsies from early, untreated, lepromatous cases. The involvement of melanophores and the variation in pigment content is of interest both in connection with the human disease and also in view of the hypothetic relationship of these cells to the Schwann cells of dermal nerves. Quite frequently, moreover, as

already mentioned, in sections in which these cells and squamous epithelium were seen to be involved, elongated dendritic processes containing mycobacteria were found in close relationship to the basal layer of the epidermis. It is possible therefore that the bacilli present in the epidermis were derived from melanophores; this would account for their relatively frequent occurrence in the former site in hamsters. In contrast, involvement of squamous epithelium in man is regarded generally as extremely rare. However, Muir and Chatterji ([24]) have reported on a human lepromatous patient whose nonulcerated skin showed an extensive invasion by acid-fast organisms of squamous epithelial cells. Indeed, Muir ([23]) has suggested subsequently that leprosy bacilli may not infrequently be present in the epidermis of patients with early lepromatous disease, with apparently normal skin. [One of us (J.S.F.N.) has observed mycobacteria in the epithelium of a hair follicle in 1 out of 4 biopsies from lepromatous cases.] The involvement of skeletal muscle is a phenomenon that we hope to investigate further. At present, as in the 2 instances of chondrocyte involvement, we feel that it may be associated with trauma at inoculation or during the observation period. The fortunate observation of bacilli in vascular endothelium is of interest, in view of the well-established reports of the occurrence of *M. leprae* in the blood in active lepromatous disease. The testis findings were less interesting. While we would emphasize that *M. leprae,* as shown by our results, must have remained viable in the hamster testis, we have no evidence of multiplication. The hamster ear is without doubt a more useful collection of tissues for experimental leprosy.

The very small numbers of bacilli detected in the 3 positive right ears of the primary inoculation series may be explained by the known persistence of mycobacteria, dead or alive, in animal tissues. The findings in hamster 40, however, cannot be thus explained. Here, although the left ear received untreated suspensions and the right ear heat-treated inoculum, the lesions on both sides were similar, and contained large numbers of acid-fast bacilli. Each tuberculin syringe was used to inoculate the corresponding side of 10 animals, the platinum needle being flamed between injections. But in no other animal inoculated from the same syringe as hamster 40 were similar lesions produced. We therefore consider that in this animal the infection had either occurred naturally in the hamster, or had been introduced accidentally at the time of inoculation, presumably from the skin surface. These suggestions would also account for the unidentified mycobacterium obtained from the left testis of hamster P11, for its right testis, inoculated at the same time with the same suspension, using the same though flamed needle, and syringe, was negative. Nishimura and his colleagues ([25, 26]) have recently reported on the spontaneous occurrence of mycobacteria in healthy mice, and hamsters. It is therefore essential that all positive suspensions should be cultured. However, many of Nishimura's isolates failed to produce growth *in vitro,* in this way

resembling our own unidentified mycobacterium. We suggest therefore that a careful study of the bacterial morphology should also be made in every case, with note of both the size and shape of the bacilli, and the percentage of solid-staining forms. All our isolates of (presumed) *M. leprae* contained a definite and usually relatively high percentage of irregularly staining bacilli, similar to the bacilli in human untreated disease ([32]). A very high percentage of solid-staining bacilli could well arouse suspicion of a *M. lepraemurium* type of intercurrent infection.

The inadequacies of our study are readily apparent, the most obvious being the failure to correlate, in the same animal, the histologic findings with bacterial counts and cultural methods. Because the bacterial multiplication is limited and the distribution of the mycobacteria is focal and irregular (see page 302), it is not practicable to use part of the same ear for histologic examination and part for a bacterial count and culture. However, a practical compromise in future studies would be to take an aliquot for culture from any specimen intended for histology, the remainder being examined in complete serial section by appropriate methods. It is only by full utilization, and careful integration, of bacteriologic and histologic methods that claims to the successful transmission of human leprosy to experimental animals can be substantiated.

SUMMARY

Forty-eight golden hamsters, inoculated in the left ear and left testis with living suspensions of *M. leprae* and in the right ear and right testis with heat-killed suspensions, were maintained for 5-22 months. Sixteen of the 23 left ears examined histologically showed typical intracellular acid-fast microorganisms in a variety of cell types. However, of 28 pairs of testes examined histologically, in only one left testis were intracellular mycobacteria found. Bacteriologically, acid-fast bacilli were recovered from suspensions prepared from 9 left testes out of 19 pairs of testes examined, and these positive suspensions were used to attempt passage to 18 hamsters. After 18 months, 6 of 8 first passage ears examined histologically showed intracellular mycobacteria in sites similar to those found in the primary inoculation animals. Homogenates from 9 other ears contained acid-fast bacilli, and counts on 4 confirmed a ten-fold increase, although the yield never exceeded 10^6 bacilli. It is concluded that a limited multiplication type of infection has been achieved in the hamster ear, but not in the testis, analogous to that described by Shepard in the mouse footpad.

RESUMEN

Durante 5 a 22 meses, fueron mantenidos cuarenta y ocho "golden hamsters," los que fueron inoculados en la oreja izquierda y testículo izquierdo con suspensiones de *M. leprae* vivos y en la oreja derecha y testículo derecho con suspensiones muertos al calor. De las

23 orejas izquierdas examinadas histológicamente diez y seis mostraron típicos microorganismos acido-alcohol-resistentes intracelulares en un variedad de tipos celulares. Sin embargo, de los 28 pares de testículos examinados histológicamente, solamente en un testículo izquierdo se encontraron micobacterias intracelulares. Bacteriológicamente, bacilos acido-alcohol-resistentes fueron recobrados de suspensiones preparadas de 9 testículos izquierdos de los 19 pares examinados, y estas suspensiones positivas fueron usadas en un intento de pasaje a 18 hamsters. Despues de 18 meses, 6 de los 8 primeros pasajes en orejas examinados histológicamente mostraron micobacterias intracelulares en lugares similares a aquellos encontrados en los animales de primera inoculación. Homogenatos de otras 9 orejas contuvieron bacilos acido-alcohol-resistentes, y recuentos en 4 confirmaron un aumento de 10 veces, aunque la producción nunca excedio los 10^6 bacilos. Se concluye que un tipo de infección multiplicada limitada ha sido conseguida en la oreja del hámster, pero no en los testículos, analogo al descripto por Shepard en la planta del raton.

RÉSUMÉ

Quarante-huit hamsters dorés ont été inoculés avec des suspensions vivantes de *M. leprae* dan l'oreille et dans le testicule gauche, et avec des suspensions tuées par la chaleur dans l'oreille et le testicule droit. Ces animaux ont été observés durant 5 à 22 mois. Seize des 23 oreilles gauches qui ont été éxaminées histologiquement ont montré des micro-organismes acido-résistants intracellulaires typiques dans des cellules de types variés. Toutefois, parmi les 28 paires de testicules, des mycobactéries intracellulaires n'ont pu être recueillis que d'un seule testicule gauche. Au point de vue bactériologique, des bacilles acido-résistants ont été recueillis de suspensions préparées à partir de 9 testicules gauches, parmi les 19 paires examinées. Ces suspensions positives ont été utilisées chez 18 hamsters afin de tenter leur passage. Après 18 mois, 6 des 8 oreilles ayant été soumises à un premier passage et examinées histologiquement ont montré des mycobactéries intracellulaires en des endroits semblables à ceux observés chez les animaux de primo-inoculation. Les suspensions homogénéisées de 9 autres oreilles contenaient des bacilles acido-résistants, et chez 4 la numération a révélé une augmentation dans la proportion de 1 à 10, encore que le nombre de bacilles recueillis n'ait jamais dépassé un million. On en conclut qu'une infection avec multiplication limitée, analogue à celle décrite par Shepard dans la sole plantaire de la souris, a été obtenue dans l'oreille du hamster, mais non dans le testicule.

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