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Transitory Macrophage Activation in the Granulomatous Lesions of *Mycobacterium lepraemurium*-induced Lepromatoid Leprosy in the Mouse[1]

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Mycobacterium lepraemurium (MLM), like other pathogenic mycobacteria, depends on the intracellular milieu of certain phagocytic cells (free and tissue-fixed macrophages) to survive and to propagate in the murine host. Paradoxically, macrophages in resistant animals, or in the case of aborted infection, are the cells responsible for the eventual killing of the microorganism. However, for this to happen the macrophages need the concourse of T lymphocytes ([11]) with a helper/inducer phenotype (Th Lc). MLM-reactive Th Lc in the presence of antigen and other soluble mediators produce lymphokines able to activate macrophages and, in so doing, increase their overall metabolism and their cidal capabilities ([6]). In the absence of MLM-reactive T cells or in the presence of excessive suppressive activity, the macrophages do not become activated, thus allowing the intracellular proliferation of the microorganism which first saturates the cell and then the tissues of the host.

Since lepromatoid leprosy in the mouse proceeds in a manner suggestive of defective cell-mediated immunity (CMI), and considering the link between CMI and macrophage activation ([13]), we decided to look for evidence of macrophage activation precisely at the site of the lepromatoid granuloma. Preliminary observations indicate that a positive biochemical activation occurs in the peritoneal mouse macrophages as a consequence of the infection with MLM ([8, 15, 16]). This biochemical activation, however, is transitory and not sufficient to arrest the progress of the disease. Other workers ([9]) have also found evidence of the early and transient enhancement of antigen- and lectin-induced mitogenic responses of lymphoid cells from MLM-infected mice and of the generation of suppressive activity ([10, 17]), which, very likely, abrogates the incipient immunity.

In this communication, we report the results of a kinetic study on the onset and evolution of lepromatoid granulomas in the mouse liver (a target organ), the process of macrophage biochemical activation within the granulomas, and the fate of MLM during the entire period of infection.

MATERIALS AND METHODS

Animals and their inoculation. Sixty albino, NIH, male mice weighing between 18 and 20 g were inoculated with MLM bacilli by the intraperitoneal (i.p.) route. A similar number of noninoculated animals served as a control group. The mice were maintained under a standard Purina chow diet and water *ad libitum* until completion of the experiments. MLM (Hawaiian strain) were separated from the lepromas of previously infected mice following Prabhakaran's procedure ([14]).

Sampling. Every 2 weeks following inoculation, three control and three infected mice were anesthetized by chloroform inhalation and then exsanguinated by cardiac puncture (blood was saved for serological tests). The spleen and the largest liver lobule were carefully excised, trimmed, and cut along the middle horizontal axis. A portion of tissue (always from the same region) was removed, embedded in O.C.T. compound (Miles Laboratories, Elkhart, Indiana, U.S.A.), frozen on dry ice, and stored at −70°C in a Revco freezer until used.

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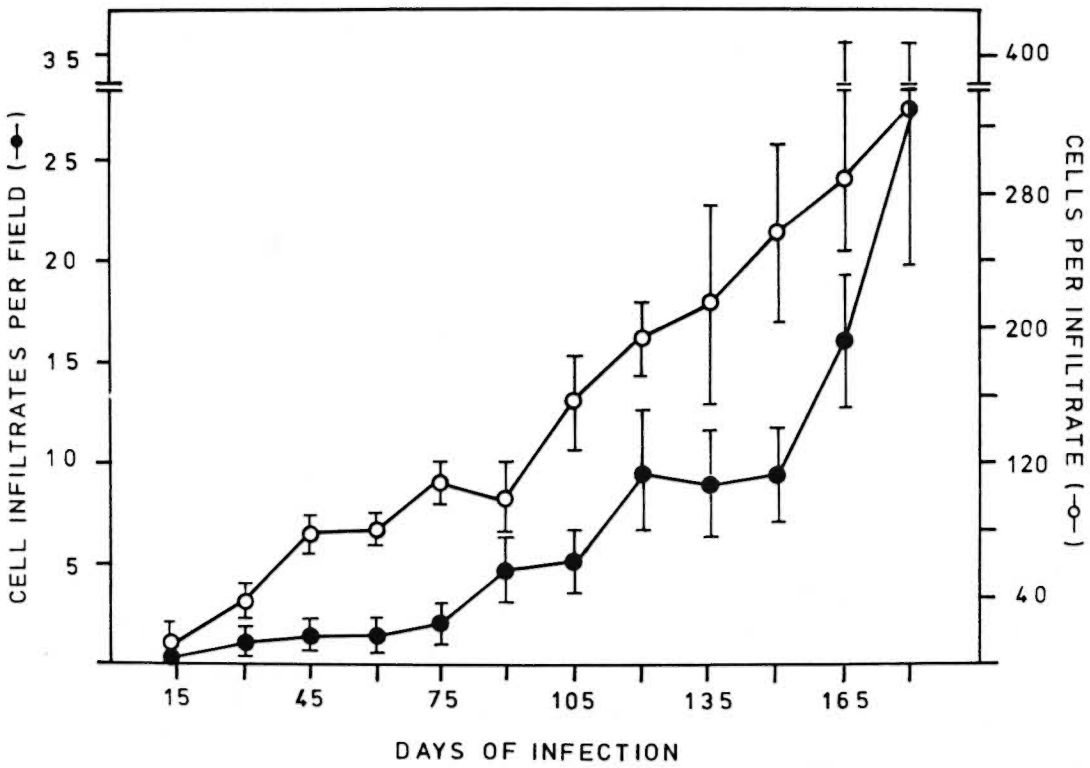

FIG. 1. Numbers and sizes of cell infiltrates in livers of mice injected with 10^8 MLM i.p. At different postinoculation times shown, three animals were sacrificed, their livers excised and processed as described under Materials and Methods. (●) = average number of cell infiltrates in 30 microscopic (12.5 × 40×) fields; (○) = average number of cells per area of infiltrate. Thirty cell infiltrates (or maximum number possible) were analyzed at a 12.5 × 40× magnification. Counts were made in triplicate tissue sections. Bars are corresponding standard deviations.

Histochemical studies. The frozen tissue specimens were cut at −20°C in a Tissue Tek II cryostat to produce 6 μm-thick sections. The sections were collected on standard glass slides, fixed for 5 min with 1.25% glutaraldehyde made in 0.15 M phosphate buffer, pH 7.2. The slides were washed three times for 5 min with 0.1 M phosphate −0.15 M NaCl (PBS), pH 7.4, and sequentially reacted with a) a substrate for β-galactosidase activity (β-Gal), b) hot phenol-fuchsin for acid-fast bacilli (AFB), and c) Harris' hematoxylin for counterstaining.

β-Galactosidase staining. ([15]) The fixed and washed tissue sections were incubated overnight at 37°C in a mixture containing 2 mg of 5-bromo-4-chloroindoxyl-β-D-galactoside dissolved in 0.5 ml of dimethyl formamide, 31 ml of 0.1 m acetate buffer, pH 5.4, 0.5 ml of 0.85% NaCl, 3.0 ml of 0.05 M potassium ferro-ferricyanide, and 8.0 mg of spermadine·3HCl.

Acid-fast staining. The slides from the previous step were gently rinsed in water and immediately stained for 10 min with the phenol-fuchsin reagent [25 ml of heat-melted phenol, 5 g of basic fuchsin, 50 ml of 95% ethanol, and distilled water (DW) to make 500 ml]. The best results were obtained when the slides were placed on a hot plate, covered with phenol-fuchsin, and heated until steam began to appear; the slides were then allowed to cool somewhat and then they were heated once more until steam began to appear, all within the 10-min staining period. After washing out the excess stain with DW, the slides were dipped for 20 sec in 1% HCl–70% ethanol to decolorize the tissue, washed again with DW, and then counterstained.

Counterstaining. The tissue sections were stained for 5 min at room temperature with Harris' hematoxylin with a 5 sec acid decoloration (1% HCl–70% ethanol), a 30 sec

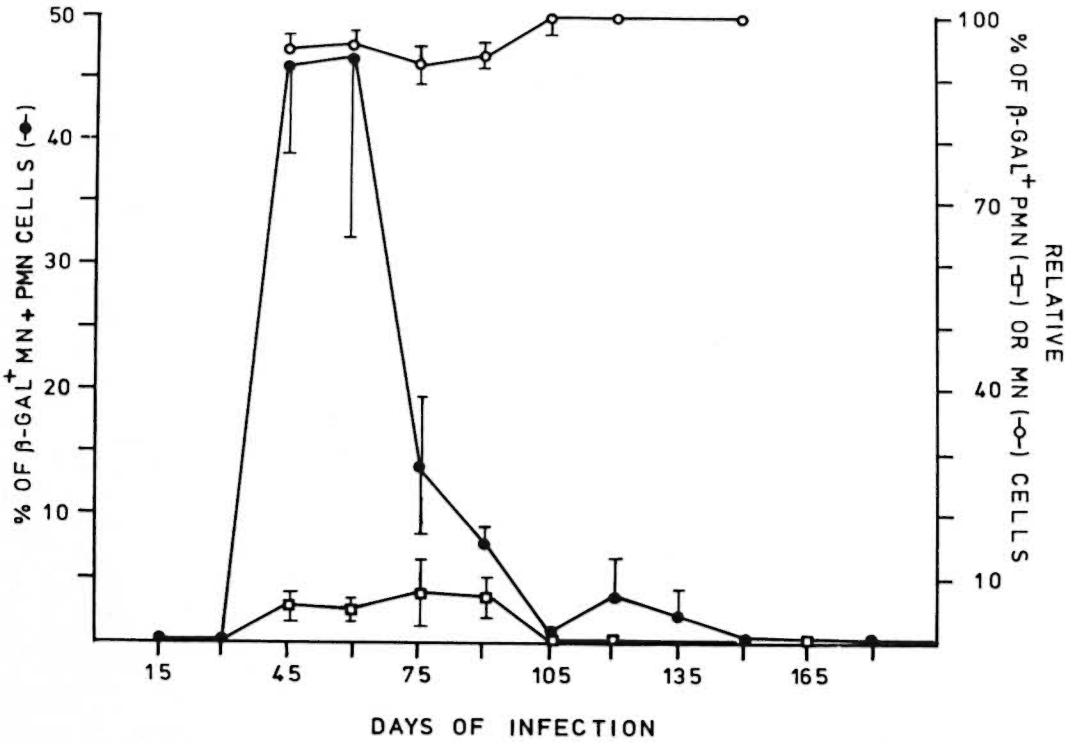

FIG. 2. Cell activation in the cell infiltrates. (●, scale 0–50%) = percent of total cells that were positive for β-Gal activity; (○, scale 0–100%) = relative percent of mononuclear phagocytes (MN) positive for β-Gal activity; (■) = relative percent of polymorphonuclear cells (PMN) positive for β-Gal activity. Cells were counted in 30 (or maximum number possible) cell infiltrates under oil (12.5 × 100×).

alkaline bluing performed by dipping slides into solution containing 2 mg $MgSO_4$/ml of 0.35% $NaHCO_3$ in water (a 0.2% $MgSO_4$ solution in 0.35% $NaHCO_3$), washing between steps with DW. The stained preparations were mounted with synthetic resin for microscopic examination.

Microscopic examination. Three slides, each containing 4 to 6 tissue slices, were prepared from each tissue fragment and stained by the process indicated above. Those preparations showing the neatest sections and staining were further analyzed, and those showing progressive pathologic changes were selected for detailed microscopic examination and description.

Cell infiltrates. The tissue sections were examined at a 40 × 12.5 magnification, and the number of discernible cell infiltrates counted in 30 microscopic fields. The size of such infiltrates was assessed both by micrometry (two perpendicular diameters) at the above magnification and by counting the number of cells in the infiltrates at a 40 × 12.5 magnification.

Cell types, bacilli, and enzyme activity. The cell types in the infiltrates and the presence and number of bacilli per area of infiltrate were determined by examining the infiltrates found in at least 30 immersion fields. Those cells showing β-Gal activity were identified and recorded. A scale from 0 to 4+ activity was arbitrarily established and used to assess the degree of biochemical activation per cell.

RESULTS

Number and size of cell infiltrates. Figure 1 shows distinct cell infiltrates in the liver parenchyma shortly after the i.p. inoculation of bacilli. The number of cell infiltrates was, however, small during the first 2.5 months postinfection but grew steadily thereafter. Beyond 195 days postinoculation, most of the cell infiltrates were confluent.

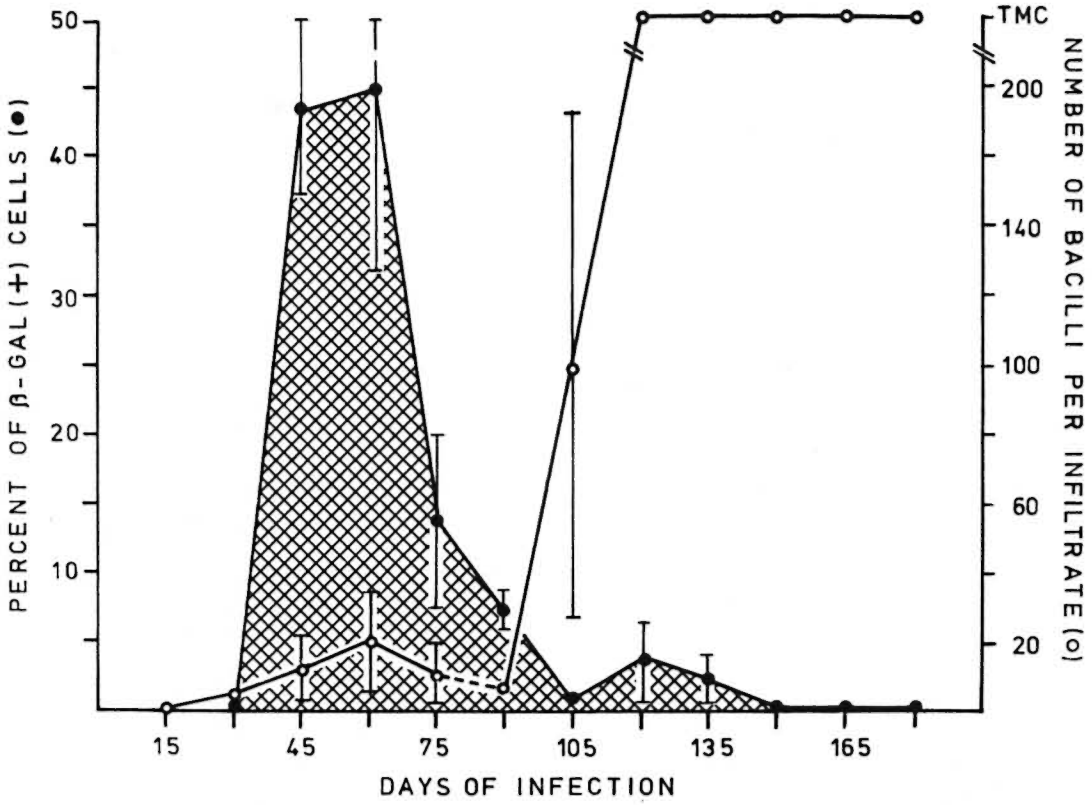

FIG. 3. Acid-fast bacilli per cell infiltrate in relation to degree of macrophage activation. (○) = average number of bacilli counted in 30 (or maximum number possible) cell infiltrates at 12.5 × 100× magnification. (– – –) between 75 and 90 days postinoculation = granular-staining bacilli were particularly predominant; (●) = percent of cells per infiltrate that were biochemically activated (β-Gal-positive macrophages).

Not only did the number of cell infiltrates grow in parallel to the time of infection but their size also increased proportionally (Fig. 1). During the early stages of the infection, the granulomas were predominantly made up of macrophages and a few scattered polymorphonuclear leukocytes (PMN). As the number and size of granulomas increased, the PMN became more abundant but they had an exclusively peripheral localization, indicating their recent arrival from the circulation.

Number of bacilli per average infiltrate area. The number of AFB per area of cell infiltrate ranged from 1 to 5 within the first 30 days postinoculation to "too many to be counted" at the end of the fourth month of infection. Since the count of AFB was made directly in the cell infiltrates, subtle changes could not be noticed. The AFB growth curve showed a bimodal profile: a small peak 60 days after inoculation that decreased to a minimal value 30 days later, and a second increment (this a very marked one) that raised the number of bacilli to unassessable levels from day 120 postinoculation onward. Concomitant to the diminution in the number of bacilli within the 60-to-90-day postinoculation period was the predominance of bacterial bodies with granular staining, this suggesting nonviable bacilli (Figs. 3 and 4).

Biochemical activation of macrophages. Macrophage activation within the granulomas was deduced from the presence and level of the hydrolitic lysosomal enzyme β-Gal ([2, 5]). Figures 2 and 4 show the early granulomas seen 15 days postinoculation which contained macrophages with no evidence of biochemical activation (β-Gal-negative). Some β-Gal-positive macrophages were found 15 days later. Their number increased, reaching a peak between 45 to 60 days postinoculation, then decreasing and

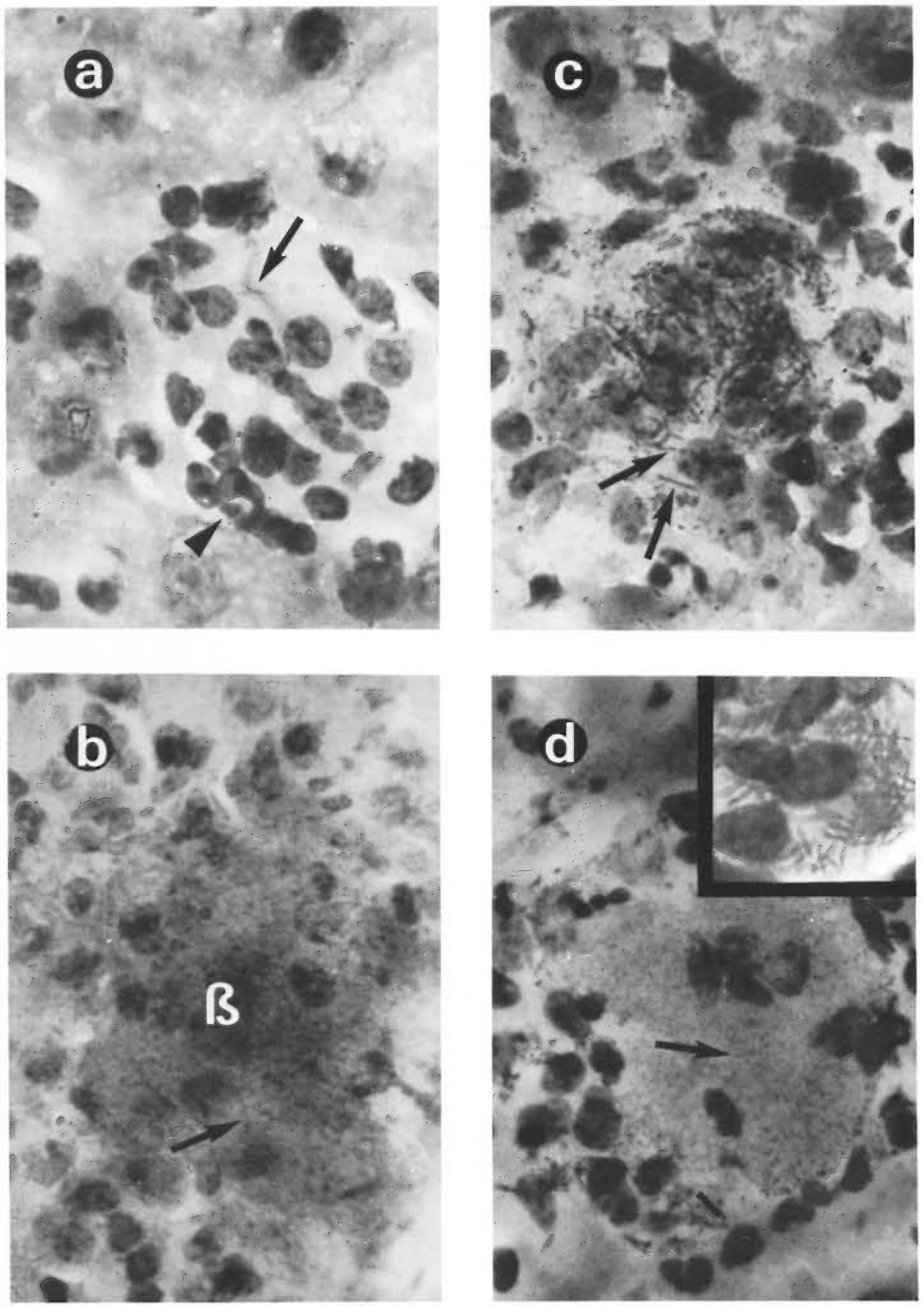

FIG. 4. Histochemical assessment of macrophage activation and bacillary multiplication.

a. A 30-day-old granuloma showing infiltrating macrophages, some PMNs (arrowhead), and several MLMs within cytoplasm of one of the macrophages (arrow). Macrophages in this early granuloma show a "clean" cytoplasm with no evidence of β-Gal activity.

b. A mature, activated granuloma, 60 days old, showing intense macrophage β-Gal activity (β) which totally or partially masks the several bacilli present (arrows).

c. A 75-day-old granuloma, showing less and more condensed β-Gal activity and several bacilli with granular staining (arrows).

d. A fully consolidated lepromatoid granuloma, 120 days old, showing macrophages heavily loaded with bacilli. Insert is a higher magnification of a similar granuloma showing presence of bacilli and absolute absence of β-Gal activity in the macrophages' cytoplasm.

practically disappearing by day 105 after inoculation. At any time investigated within the peak period, there were some (less than 10%) PMNs showing some β-Gal activity (Fig. 2, The Table).

Other histologic changes included some vacuolation of the hepatocytes noticeable from day 45 postinoculation but no inflammatory reaction (cell infiltration in the normal liver tissue), the presence in the granulomas of lymphocytes, some epithelioid and giant cells between 30 and 75 days postinfection, and a predominance of histiocytes thereafter. As mentioned above, PMNs were always present, scarce in the early lesions but abundant in the well-conformed granulomas where they showed a peripheral localization. Despite the fact that PMNs are highly phagocytic and bactericidal cells, they do not seem to efficiently participate in the control of the lepromatoid disease caused by MLM in the mouse. Perhaps this is due to the following circumstances: a) the PMNs that circulate and enter the tissues are mature cells with a relatively short life span (24 to 42 hr [1]), and b) MLM has a long replication time (9 ± 1 days) and a very complex lipidic envelope ([7]) which makes it particularly resistant to intracellular digestion.

DISCUSSION

This histochemical and bacteriologic study of the granulomas that appear in the liver of mice infected with MLM reveals that, despite the large number of bacilli used for infection, each lesion begins with the arrival of isolated phagocytic cells (namely, macrophages) containing from 1 to 5 bacilli. These moderately parasitized phagocytes reach the liver parenchyma through the blood circulation so that the small granulomas that appear early in the infection have a predominantly perivascular distribution. Bacilli within the phagocytic cells, or the parasitized phagocytes themselves, must produce potent chemotactic factors that attract fresh phagocytes to the site of infection with the kinetics depicted in Figure 1. Macrophages within the granulomas become biochemically activated due to their normal maturational process, to the presence and endocytosis of bacilli, or in response to macrophage-activating lymphokines generated during the early stages of the infection. On the average, the biochemical activation of macrophages in the liver granulomas, as assessed by their levels of β-Gal, an enzyme whose activity increases proportionally to the degree of cell activation ([4, 5]), becomes evident around 30 days after inoculation. It then increases relatively rapidly to reach a peak between 45 and 60 days, decreasing thereafter and completely disappearing by day 150 postinfection (Figs. 2 and 4). Within the granulomas the bacilli seem to multiply steadily during the first 60 days of infection, then their growth seems to be arrested and granular bacilli become evident. This period of stasis and/or killing of bacilli lasts for some days (around 2 weeks), and it is followed by a period of logarithmic bacterial growth that continues until saturation of the tissue occurs (Figs. 3 and 4). Within this period of logarithmic growth, the mean generation time was 9 ± 1 days.

These findings on the *in situ* macrophage inactivation that ensues and vanishes during the course of the lepromatoid infection of the mouse support and complement those from a previous study ([15]) in which the levels of several lysosomal enzymes were quantitated in the peritoneal cell population of mice infected with MLM. In that study, it was observed that most of the assayed enzyme activities showed a progressive elevation that peaked between 40 and 75 days postinfection. There was a tendency for these enzyme activities to decrease thereafter until the end of the experiment (over 6 months postinoculation), when most animals showed obvious signs of the disease that eventually killed them.

There is the possibility that the increased growth rate of bacilli in the liver might be due to an overflow (by redistribution) of bacilli from other compartments ([2]), and that we might be confronted with immunological exhaustion rather than active suppression. We favor the latter possibility because: a) several groups have found evidence in support of the generation of a cell-mediated immune response following the inoculation of mice of several strains (CB6, C57BL/6, CBA, and BALB/c) with MLM ([2, 9, 10, 12, 17]); b) in most of these studies, the MLM-elicited CMI has also been found to be transitory ([2, 9, 10, 17]); and c) in some cases, Lyt 1+2− and Lyt 2+ T cells ([10]) or nylon-wool adherent spleen cells ([2, 17]) have been found

THE TABLE. *Some histologic, bacteriologic, and biochemical changes in the livers of mice infected with* Mycobacterium lepraemurium.[a]

	Days of infection											
	15	30	45	60	75	90	105	120	135	150	165	180
No. cell infiltrates per field[b]	0.2[c]	1.2	1.5	1.4	2.0	4.9	5.0	9.9	9.1	9.3	16	28
No. infiltrating cells per area of infiltrate[d]	16	39	85	86	114	102	161	196	218	263	292	339
% β-Gal(+)[e] total cells per area of infiltrate	0	0	46	47	14	8	1	4	3	0.5	0	0
Relative % of β-Gal(+)[e]	0	0	94	95	92	93	100	100	100	100	0	0
MN cells	0	0	6	5	8	7	0	0	0	0	0	0
PMN cells												
No. bacilli per area of infiltrate[f]	1	5	13	21	11	8	99	TMC	TMC	TMC	TMC	TMC

[a] Each animal received 1.0×10^8 bacilli i.p.
[b] Thirty microscopic ($12.5 \times 40\times$) fields in triplicate tissue sections were investigated.
[c] Mean values, standard deviations not given but are shown in the figures.
[d] Cells in 30 cell infiltrates were counted; when infiltrates were scarce all of them were counted in triplicate tissue sections ($12.5 \times 40\times$).
[e] Cells that were positive (1+ to 4+) for β-gal activity were counted in 30 (or maximum number possible) cell infiltrates under oil ($12.5 \times 100\times$).
[f] Acid-fast bacilli were counted in 30 (or maximum number possible) cell infiltrates ($12.5 \times 100\times$); TMC = too many to be counted.

to exert the suppressive activity on the MLM-induced cellular immunity.

The presence and initial proliferation of bacilli in the host tissues very likely stimulates MLM-reactive, T lymphocytes (Th) which, through lymphokines, activate the macrophages of the incipient granulomas, increasing their metabolic and microbicidal functions. Theoretically, this activation should be enough to render the macrophages able to destroy the still scant local bacilli. However, when activation vanishes (whatever the reason might be), the few bacilli surviving in the lesions, or those bacilli arriving from infected sites lacking lymphoid tissue (bacilli that have not be subjected to the CMI-dependent microbicidal effects), now in the absence of macrophagic resistance, start growing within the granuloma-resident macrophages and within the recently arrived histiocytes. The macrophagic granulomas extend their volume and fuse with each other to eventually replace the otherwise normal tissue in the target organs which, for this reason, become nonfunctional (Fig. 4). This can account for the immunological (and other) disturbances frequently reported in advanced murine and human leprosy.

Proof that activated (β-Gal-positive) macrophages have increased microbicidal and digestive power comes from Dannenberg's group ([4, 5]). By autoradiography, they found that intact ^{14}C-labeled tubercle bacilli were very rare in macrophages staining 4+ for β-Gal yet these cells frequently contained ^{14}C-associated bacillary breakdown products. The macrophages staining 1+, 2+, and even 3+ for β-Gal frequently contained both intact bacilli and, of course, ^{14}C-labeled bacillary fragments.

SUMMARY

A kinetic study on the evolution of granulomas that appear in the liver of NIH mice inoculated with 10^8 *Mycobacterium lepraemurium* by the intraperitoneal route has been performed. The liver was chosen because of its nonlymphoid histology which allowed us to visualize the appearance and maturation of the cell infiltrates generated as a consequence of the mycobacterial infection. The study analyzed both the macrophage activation within the granulomas and the fate of bacilli within the macrophage. The results showed that this mycobacteriosis induces a relatively early macrophage activation (a very likely result of a

cell-mediated immune response triggered by the bacilli) that peaks between 45 and 60 days postinoculation, fades thereafter, and practically disappears several days later. Bacilli are susceptible to the microbicidal effects of activated macrophages, but when the macrophages are turned off (probably due to active suppressive mechanisms), the surviving bacilli reinitiate the infection with no further macrophage opposition. As a result, more phagocytes are attracted to the infection sites and the cell infiltrates grow steadily to become confluent, increasing the granuloma fraction and eventually replacing the liver parenchyma. The findings suggest that in murine "leprosy" infection, early immunological changes occur that enable the macrophages present in the granulomas to kill the infecting *M. lepraemurium* regardless of the eventual lepromatoid evolution of the granulomas. Lepromatoid granulomas in the mouse and lepromatous granulomas in man are equivalent structures in regard to their histology and bacteriology.

RESUMEN

Se hizo un estudio cinetico sobre la evolución de los granulomas que aparecen en el hígado de ratones NIH inoculados con 10^8 *Mycobacterium lepraemurium* por la vía intraperitoneal. Se escogió el hígado porque su histología no linfoide nos permite visualizar la aparición y maduración de los infiltrados celulares generados como consecuencia de la infección micobacteriana. En el estudio se analizó tanto la activación de macrófagos dentro de los granulomas como el destino de los bacilos dentro de los macrófagos. Los resultados mostraron que esta micobacteriosis induce una activación de macrófagos relativamente temprana (muy probablemente resultante de una respuesta inmune celular contra los bacilos) que alcanza su pico entre los 45 y 60 días post-inoculación y que disminuye progresivamente hasta prácticamente desaparecer varios días más tarde. Los bacilos son efectivamente susceptibles a los efectos microbicidas de los macrófagos activados pero cuando los macrófagos son desactivados (debido, probablemente, a mecanismos supresores activos), los bacilos sobrevivientes reinician la infección sin posterior oposicíon macrofágica. Como resultado de esto, más fagocitos son atraídos a los sitios de infección, los infiltrados celulares crecen sostenidamente hasta hacerse confluentes, aumentando la fracción granuloma y finalmente substituyendo a la mayor parte del parenquima hepático.

Los hallazgos sugieren que en la infección leprosa del ratón, ocurren cambios inmunológicos tempranos que capacitan a los macrófagos de los granulomas para matar al *M. lepraemurium* aún cuando la infección termine en su forma lepromatoide, una forma equivalente a la lepromatosa del humano en cuanto a histología y bacteriología.

RÉSUMÉ

On a mené une étude cinétique de l'évolution des granulomes qui apparaît dans le foie des souris NIH inoculées par 10^8 *Mycobacterium lepraemurium* par voie intrapéritonéale. Le foie a été choisi pou ces études, car son histologie non lymphoïde permet de rendre visible l'apparence et la maturation des infiltrats cellulaires qui se développent à la suite de l'infection mycobactérienne. Cette étude a analysé à la fois l'activation des macrophages à l'intérieur des granulomes, et le sort des bacilles dans les macrophages. Les résultats ont montré que cette mycobactériose entraîne une activation relativement précoce des macrophages, résultat probable de la réponse immunitaire à médiation cellulaire produite par les bacilles. Les pics se situent entre 45 et 60 jours après inoculation, s'estompent ensuite, et disparaissent pratiquement quelques jours plus tard. Les bacilles sont vulnérables aux effets microbicides des macrophages activés; néanmoins, lorsque les macrophages ne sont plus activés, probablement à la suite de mécanismes actifs de suppression, les bacilles qui survivent reprennent le relais sans aucune défense ultérieure par les macrophages. En conséquence, davantage de phagocytes sont attirés vers les sites d'infection, et les infiltrats cellulaires se développent sans obstacle jusqu'à ce qu'ils deviennent confluents, ce qui entraîne une augmentation de la proportion de tissu granulomateux, qui en vient à remplacer le parenchyme du foie. Ces observations suggèrent que dans l'infection par la lèpre murine, des modifications immunologiques surviennent précocement, permettant ainsi aux macrophages présents dans les granulomes de tuer les *M. lepraemurium* responsables de l'infection, et ceci sans égard à une évolution lépromatoïde éventuelle des granulomes. Les granulomes lépromatoïdes chez la souris, de même que les granulomes lépromateux chez l'homme, constituent des structures équivalentes sur le plan histologique et sur le plan bactériologique.

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