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Intracellular Modulation in Cellular Immunity

I. Morphologic Studies of Macrophages in Murine Leprosy Under Conditions of Immunity Enhancement and Suppression.[1,2]

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Cellular immunity has been shown to be the major defense mechanism of the body against chronic infections where the pathogens are facultative or obligate intracellular parasites ([22, 36]) whereas serum antibodies, when present in such diseases as tuberculosis and leprosy, do not provide protection ([11, 26, 29, 37]). It has been postulated that in leprosy, as well as in other disseminated (lepromatoid) forms of chronic granulomatous disease a major immunologic defect is related to an inherent inability of the macrophages to digest the pathogen concerned, and to concomitant inability to develop an enhancement of such a function ([32]). The discovery of the intracellular "digestive system," lysosomes, which are rich in hydrolytic enzymes ([5]), has opened a broad field relative to the role of cell enzymes in cellular immunity. The known nature and functions of these cellular organelles have been recently reviewed by Hirsch ([16]) and Straus ([35]). Their possible significance in macrophage digestion of mycobacterial infectious agents is, however, not clear. Murine leprosy offers a useful disease model for eliciting information concerning intracellular modulation of cellular immunity because of the obligate intracellular parasitism and long generation time of *M. lepraemurium* ([4]) as well as the long duration of a mild and progressive interaction between macrophage and pathogen. Therefore, information derived from studying this disease model is particularly useful in developing an understanding of the complicated immunologic phenomena of human leprosy.

The present series of studies, utilizing murine leprosy as a model and applying histologic, electron microscopic, histochemical, chemical, radioautographic and EM-radioautographic technics, were designed to explore intracellular modulations in macrophages in immunized, debilitated (hypoproteinemic) and control animals in the early stages of development of immunity during the course of infection.

MATERIALS AND METHODS

Experimental animals. *M. lepraemurium* susceptible C_3H strain, inbred female mice were used. They were seven to 10 weeks old on experimental initiation but in any

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given experiment they were of the same age. The mice were fed Purina chow and water *ad libitum*, except as indicated in experiments involving nutritional debilitation, and were housed in groups of five per stainless steel cage.

Bacillary inoculum. The Hawaiian strain of *M. lepraemurium* was used and obtained from lepromas of stock infected mice. The lepromas were minced and homogenized to a smooth suspension in normal saline in a sterile Potter-Elvehjem homogenizer. This crude suspension was centrifuged at 1,500 rpm (Servall SS-4 centrifuge, GSA rotor) for 20 minutes to remove tissue fragments and clumps of bacilli. The supernatant fluid was then recentrifuged at 5,000 rpm for 20 minutes and the supernatant fluid discarded. The sediment was resuspended and again centrifuged at 5,000 rpm for 20 minutes. This latter washing procedure was repeated a total of three times and the final sediment was then resuspended in saline and adjusted to yield not less than 10^7 bacilli per milliliter.

Bacillary counting. The bacillary suspension was diluted to about 10^7 bacilli/ml. and mixed in a 1:1 volumetric ratio with 1 per cent methylene blue solution. After standing for at least three hours at room temperature, the bacilli were counted in a hemocytometer. A double check of the count was provided by recounting the bacilli when the chamber solution had dried and the bacilli had settled on the chamber plate. This is a simple and accurate counting method if the bacillary suspension is purified. A high degree of accuracy was established by checking against the more complicated method described by Hart *et al.* ([13]) and by using *M. phlei* as control. The latter can be readily cultured on available media and the accuracy of the counting method checked by pour-plate colony counts.

***M. lepraemurium* antigen and Freund's adjuvant.** Heat-killed *M. lepraemurium* bacillary suspensions containing 10^8 bacilli/ml. were twice subjected to French pressure cell processing at 6,500 pounds pressure per square inch. This antigen then showed almost no solid staining acid-fast organisms. Freund's complete adjuvant (Difco) and the antigen were emulsified together in a 1:1 volume relationship before injection.

Protein depletion. The mice were fed protein-free diet[4] and water *ad libitum* and were housed in groups of five per stainless steel cage. The body weight was recorded weekly. Blood was drawn from the heart at the time of sacrifice for determination of serum protein which was done by the Lamotte falling drop densitometer method ([3]) and calculated from the Moore and Van Slyke formula ([23]).

Peritoneal cell collection. Experimental mice were sacrificed by cervical spinal dislocation. The peritoneal lining was exposed and 2 ml. normal saline were injected into the peritoneal cavity. After gentle massage for one minute the peritoneal contents were withdrawn by means of a pipette introduced through a small peritoneal incision. Smears of the peritoneal cell suspension were made on glass slides, incubated in a 37°C humid chamber for 15 minutes, and dried by blowing air over the slides to avoid salt crystal formation. Slides were stained with Ziehl-Neelsen and Giemsa stains.

Light microscopy. The viscera were fixed in 10 per cent buffered formalin while sternums were fixed in formol-Zenker fixative. Sectioned tissues were stained with hematoxylin-eosin, Ziehl-Neelsen, and Triff ([38]) stains. Sternums were decalcified before trimming and embedding. The sections were carefully examined for acid-fast organism and scored for bacillary content. Omentums and mesenteries were spread on glass slides, dried, fixed in 10 per cent buffered formalin and stained with Ziehl-Neelsen stain.

Electron microscopy. Samples of spleen, liver and peritoneal cells from infected animals were utilized for electron microscopy (EM). The peritoneal cell suspensions were centrifuged at 1,000 rpm for 10 minutes and the resulting cell pellets were fixed in the same manner as the other tissues, i.e., for one hour at 5°C in buffered osmic acid. The tissues were then dehydrated in graded alcohols and embedded in

[4] Prepared by General Biochemicals, Laboratory Park, Chagrin Falls, Ohio.

Epon. Thin sections were cut on a Porter-Blum MT-2 ultramicrotome with glass knives and collected on 200 mesh copper grids covered with Formvar film. Staining included uranyl acetate and Reynold's lead citrate ([27]). Specimens were examined and micrographed with an RCA, EMU 3c electron microscope.

EM histochemistry. Peritoneal cell pellets and small slices of tissue selected for EM histochemistry were fixed overnight in 4 per cent cold formol-calcium solution in 5 per cent sucrose solution ([10]). They were then washed in 0.05 M acetate buffer at pH 5 for five minutes and incubated at 37°C Gomori staining medium ([8]) for one hour. Again they were washed in acetate buffer and fixed in osmic acid for one hour. After washing in 70 per cent ethyl alcohol several times, the tissues were finally subjected to the routine embedding, cutting and staining procedure noted above.

RESULTS

Effect of immunization on host survival in lepraemurium infection. It has been suggested ([12, 19]) that a vaccine prepared from *M. lepraemurium* will protect mice from rapid progression of the infection and will decrease the growth rate of subcutaneous lepromas induced by this same mycobacterium. In our experimental model, the validity of such observations was tested by an examination of survival rates of immunized animals as compared with non-immunized controls.

For purposes of obtaining a better understanding of the experimental disease model, for inoculum dose standardization, and for establishing survival curves for comparison with the effects of immunization, a preliminary experiment was designed to establish some understanding of the relationship of the *M. lepraemurium* inoculum size to host survival. A total of 140 mice were employed and so divided that each group had 20 to 25 animals. Two routes of inoculation, intravenous and intraperitoneal, were employed. For each of these routes, three groups of animals were given 10^7, 10^8 and 10^9 *M. lepraemurium* bacilli respectively and the survival number and percentage were scored at three-month intervals. The results are presented in Figure 1.

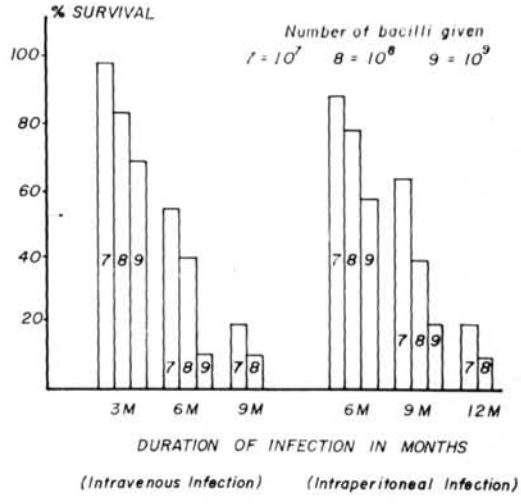

FIG. 1. Pathogen inoculum versus host survival. Two routes of inoculation, intravenous and intraperitoneal, were employed. For both of these routes, three groups of animals were given 10^7, 10^8, and 10^9 *M. lepraemurium* bacilli respectively and the survival number and percentage were scored at three-month intervals. Each group had 20 to 25 animals.

It was noted that the animals receiving the lower inoculum concentrations had a survival advantage and that the intraperitoneal route of infection provided a significantly longer survival time than did the intravenous channel. At all doses used, there was 100 per cent morbidity and eventual mortality. Therefore an inoculum of 10^7 bacilli was adequate for producing infection in every animal. The intraperitoneal route was considered preferable because of the ease of inoculation, less overwhelming infection, and the possibility for study of the localizing effect of immunization by comparative demonstration of the extent of lesion dissemination.

The 40 animals used for studying the effect of immunization were divided into two equal groups. One group was given one ml. subcutaneously of the emulsified *M. lepraemurium* and complete Freund's adjuvant (1:1 vol. ratio) at four different sites on the back. The adjuvant control group of 20 animals was similarly given 1

ml. of an emulsion of complete Freund's adjuvant emulsified with saline in a volume ratio of 1:1. Four weeks after the immunization procedures, all animals were challenged intraperitoneally by 10^7 *M. lepraemurium* in a 0.5 ml. saline suspension. Animal survival was recorded at three-month intervals and compared with the results of the nonimmunized, infected control group. The comparison is shown in Figure 2. Survival rate differences were subjected to the X^2 test and the prolonged survival time of immunized as compared to control animals was found to be statistically significant. The complete Freund's adjuvant alone did not enhance resistance.

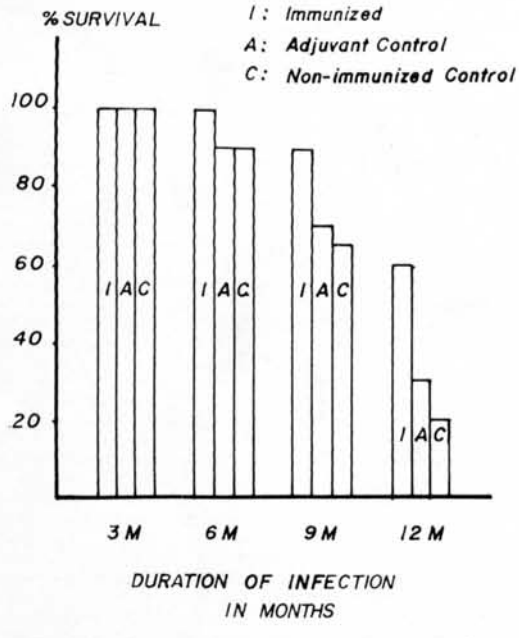

FIG. 2. Comparative survival rates of immunized (I), nonimmunized (C) and adjuvant control (A) animals. Each group had 20 animals which were infected by intraperitoneal route with 10^7 bacilli.

Further examination of the results indicates that the immunization procedure did not prevent ultimate demise of the infected animals. The prime effect was to give the immunized animals the benefit of a degree of immunization sufficient to suppress initial dissemination of the infection. Presumably, the nonimmunized mice, after about four weeks of active infection, developed the same degree of immunity possessed by the initially immunized animals. Thereafter the difference in the course of the infection was primarily that of the effect of the vaccine-induced lag phase of the infection so that at any given time the nonimmunized animals had a greater replicating bacillary content and dissemination and, therefore, a more rapidly progressing infection.

Immunosuppressive effects of protein depletion. The method used to demonstrate the immunosuppressive effects of protein starvation on C_3H mice was patterned largely after the method employed by Skinsnes and Higa ([33]).

As shown in Table 1, the protein-free diet produced a significant decrease in animal weights, serum proteins, peritoneal cell counts, and spleen size over a period of six to 10 weeks. Eight out of 40 mice on the protein-free diet died in a cachectic state after six weeks. In this preliminary study, 10 protein-starved animals were sacrificed at three weeks; 10 at six weeks, and 12 at 10 weeks. A marked decrease of serum protein from 6.06 ($\pm$ 0.18) gm./100 ml. to 4.18 ($\pm$ 0.19) gm./100 ml. was noted in mice sacrificed at six weeks. At 10 weeks the 12 animals sacrificed were severely debilitated, having a serum protein as low as 4.02 ($\pm$ 0.13) gm./100 ml. The control animals showed, over a period of 10 weeks, no significant change in their serum protein, peritoneal cell counts or spleen size, and gained an average of 7 gm. in body weight.

In Experiment No. 1, the protein-starved animals were on the diet for six weeks and then were given intraperitoneal infection with 10^7 bacilli. Five control animals were similarly infected and sacrificed along with the five protein-starved animals, and all were sacrificed according to the schedule employed in Table 2. (These five animals were the only survivors from a group of 30 mice originally begun on the protein-free diet.) The bacillary distribution in the viscera (Table 2) was determined by light microscopic examination of the Ziehl-Neelsen stained tissue sections under oil immersion lens (950X). At least 20 fields were carefully searched for acid-fast bacilli in each section and the average number of

TABLE 1. *Effects of protein-free diet on animal body weight, serum protein, peritoneal cell count and spleen weight.*

	Duration on experimental diet			
	Initial	3 weeks	6 weeks	10 weeks
Body weight (gm.)	15	12	10	10
Serum protein (gm%)	6.06 (±0.18)	4.88 (±0.16)	4.18 (±0.19)	4.02 (±0.13)
Peritoneal cell count (mm^3)	2.25×10^3 ($\pm 0.40 \times 10^3$)	1.0×10^3 ($\pm 0.25 \times 10^3$)	0.65×10^3 ($\pm 0.20 \times 10^3$)	0.5×10^3 ($\pm 0.15 \times 10^3$)
Spleen weight (gm.)	0.105 (±0.018)	0.038 (±0.008)	0.027 (±0.011)	0.019 (±0.003)

Each figure is the average of 10–12 animals.

TABLE 2. *Bacillary distribution in viscera of animals receiving protein-free diet and normal diet. (Intraperitoneal infection).*

Number of animals	Diet	Duration on diet before infection (weeks)	Duration of infection (weeks)	Liver	Spleen	Lung	Kidney	Bone Marrow
			(Experiment 1)					
5	Protein-free	6	4	1+	2+	—	—	—
5	Normal diet	6	4	—	—	—	—	—
			(Experiment 2)					
5	Protein-free	0	6	—	1+	—	—	—
5	Normal diet	0	6	—	—	—	—	—
5	Protein-free	0	10	3+	3+	1+	1+	1+
5	Normal diet	0	10	1+	2+	—	—	—

Each + increment equals a ten-fold increase in bacilli.

bacilli were recorded. After four weeks infection the protein-depleted animals already had noticeable numbers of bacilli in liver and spleen sections, while similar section from the control group showed no demonstrable bacilli.

Experiment 1 indicated that a large percentage of the mice that had been on protein-free diet could not survive longer than four weeks of infection. A second study (Experiment 2) was modified so as to prolong the duration of infection. To this end the protein-free diet was begun concomitantly with *M. lepraemurium* infection. Five animals in each group were sacrificed and examined at six and 10 weeks after infection. The scored bacillary content in the tissue sections here also indicated significant differences between animals on normal diet and animals on protein-free diet. It was also noticed that even at 10 weeks of infection, the control animals were still bacteriologically negative in lung, kidney and sternal bone marrow sections. There were obvious differences between these results and those previously

reported in protein-depleted rats ([33]). These differences were apparently due to the difference in size of inoculum utilized and differences in relative susceptibility of the species. Nevertheless, the comparable groups of protein-depleted animals all showed a wider and earlier dissemination of bacilli, involving more organs than did the comparable control animals.

Comparative cellular responses of murine leprosy infections in immunized, protein-depleted and control animals. Comparisons were made by light and electron microscope examinations of peritoneal cell preparations, mesenteric and omental spreads, and tissue sections from the three groups of animals sacrificed at the intervals indicated in Tables 3 and 4.

Light microscopic examination. The early cellular response in control infected animals, was a rapid increase in the number of peritoneal phagocytes, including polymorphonuclear leucocytes and macrophages as shown by the cell count of the peritoneal washings (Fig. 3). It rose sharply from 2,250/cu.mm. to 3,800/cu.mm. in the first four hours. This increase consisted mainly of polymorphonuclear leucocytes. After three days of infection, the percentage of granulocytes dropped rapidly but the number of mononuclear (macrophage) phagocytes increased steadily up to 15 days and then reached a plateau. Cell counts of peritoneal exudate of the immunized animals presented a similar result but the

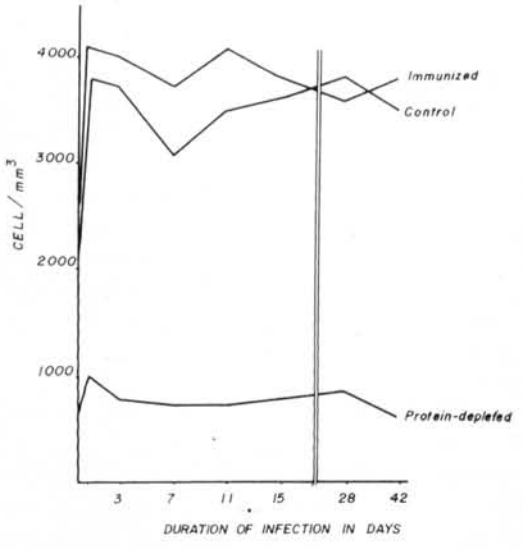

FIG. 3. Total peritoneal cells of immunized, protein-depleted and control animals.

TABLE 3. *Time of sacrifice and number of animals examined. (For peritoneal cell and tissue histopathologic examination).*

Animal group	Hours after infection		Days after infection					
	0	4	3	7	11	15	28	42
Control	4	4	4	4	4	4	4	4
Protein-starved	4	4	4	4	4	4	2	2
Immunized	4	4	4	4	4	4	4	4

TABLE 4. *Time of sacrifice and number of animals examined. (For EM and EM histochemical studies).*

Animal groups	Days after infection					Months after infection		
	0	1	7	15	28	3	4	5
Control	2	2	2	2	2	2	2	2
Protein-starved	2	2	2	2	2	0	0	0
Immunized	2	2	2	2	2	2	2	2

average level remained higher than the control-infected animals. Protein starved animals, on the other hand, showed a marked reduction in cell quantity, presenting less than 1,000 cell/cu.mm. as contrasted with the other groups which maintained levels of about 3,500/cu.mm. This was a more striking difference between the protein-starved and control animals than Azirvadham ([2]) reported for total peripheral blood leucocyte counts in protein-depleted and control rats. In his rats, stimuli such as subcutaneous turpentine, resulted in approximately 25 per cent less leucocyte response in depleted animals as compared to controls, whereas the peritoneal exudates of the protein-starved mice in the present study reflected a total cell count of 75 per cent less than the total presented by the control and immunized groups. The peritoneal cell count, reflecting the status at the site of inflammation, may thus be a more significant measure of debilitation than the peripheral blood leucocyte count. Peripheral blood leucocyte counts in the mice of these experiments fluctuated and varied so widely as to preclude evaluation and are not reported here.

The percentage of polymorphonuclear leucocytes in the peritoneal exudate of all three experimental groups paralleled each other with remarkable fidelity through the total course of the experiment, beginning at about 40 per cent of the total count and declining rapidly during the first seven days to about 10 per cent and then maintaining this level thereafter. This constancy and the relatively small percentage of granulocytes in the three experimental groups made significant the subsequent comparisons of macrophage cell enzyme content despite the mixed cell population of the system ([42]).

The double peak curves of total peritoneal cell counts (Fig. 3) in all three groups reflected the shifting from a peak of early polymorphonuclear leucocyte counts to a gradually increasing macrophage proportion. This increase in macrophages was more rapid in the immunized animals than in the control animals. The protein-depleted animals showed only insignificant increase of macrophages as late as 28 days and then dropped to the level of the initial state by the 42nd day.

Phagocytosis took place immediately following the infection and the bacilli were found in polymorphonuclear leucocytes and macrophages of all three groups of animals as shown by preparations of peritoneal cell smears. The immunized animals appeared to have more large macrophages with rich cytoplasm than the control groups. These large macrophages usually formed small clusters that often trapped and phagocytized many bacilli. By the 3rd day of infection, there also was evident phagocytosis of polymorphonuclear leucocytes by macrophages. This phenomenon was more frequently encountered in cell smears of immunized animals than in the controls.

The macrophages in protein-starved animals seemed to be smaller and lighter in cytoplasmic staining than the macrophages of the controls and those immunized. Following infections there were, in the smears, more broken and squashed cells in the protein-starved group than the others, although all preparations were processed in the same manner. A general impression was that these cells (protein-starved macrophages) were more fragile than usual.

The omental and mesenteric spreads were used to demonstrate not only the reaction of the free wandering phagocytes that settled on these membranes but also the important reactions of the vascular system and the reactions of the fixed macrophages of the reticuloendothelial system. The quantitative difference of cellular responses between control and immunized groups shown in the peritoneal cell counts were not easily differentiated in this examination. The preparations from both the control and immunized animals taken at four hours, showed similar increases in both polymorphonuclear and mononuclear phagocytes and both cell types already contained phagocytosed bacilli. There were also small foci of phagocytes, packed closely together, entrapping the pathogens by surface phagocytosis and intersurface phagocytosis ([40, 41]). These were evident in both the immunized and control animals. A

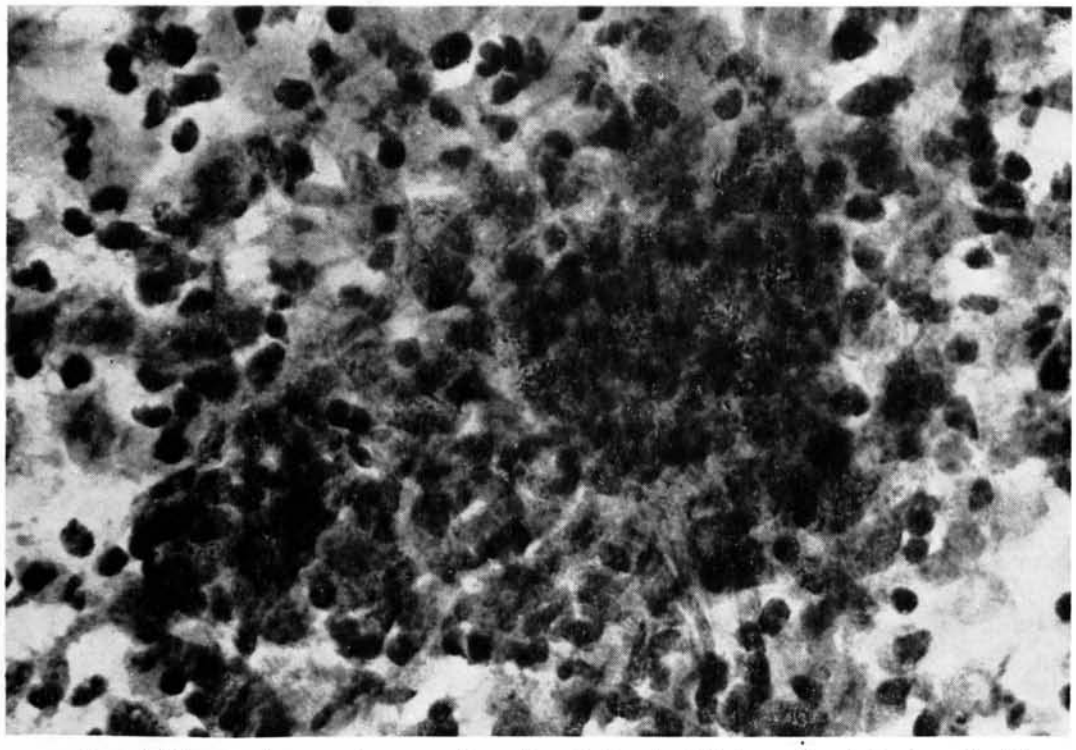

FIG. 4. Mesenteric spread preparation of control animal 7 days after infection. Bacilli laden macrophages are surrounded by many epithelioid cells to form a leproma. Ziehl-Neelsen stain, magnification, 400X.

smaller proportion of small mononuclear cells (lymphocytes) were present and usualy did not give evidence of phagocytosis of bacilli. At the seventh day of infection the granulocyte reaction subsided leaving mainly mononuclear cells around the bacilli-laden macrophages and developing into small granulomas (Fig. 4). In protein-starved animals, owing to the paucity of cell response to the acid-fast bacilli these "focal" lesions were rare. By days 17 to 28 of the infection the lesions that were near the capillary and/or lymphatic channels began to cause damage to the endothelial cells and therefore the vessel walls. This was clear demonstration of how, at this stage of infection, a large number of bacilli began to spread to the viscera.

The course of events in the protein-starved animals contrasted strikingly with that in the two groups just described. Though not absent, the inflammatory response reflected clearly the lesser availability of inflammatory cells as revealed by the peritoneal cell counts. There did not appear to be any less phagocytosis on the part of the granulocytes and macrophages that were present but there were markedly fewer reacting cells and much less accumulation of cells around bacilli and bacilli-laden macrophages. Whereas in the other two groups damage of endothelial cells of capillaries was not noted till 17 to 28 days, in the protein-starved animals there were a bacilli prominently present in the endothelial cells by the seventh day (Fig. 5). Dissemination of bacilli to the viscera occurred much earlier as compared with the control and immunized groups.

This pattern of phagocyte response to mycobacteria, under conditions of debilitation by protein starvation, contrasts with that reported by Wissler ([39]) and by Skinsnes ([31]) in the inflammatory response to pneumococcus infection in the presence of protein depletion in rabbits and rats. In the acute pneumococcus infection there was significant diminution of phagocyte activity, apparently secondary to the decrease in humoral antibodies such as opsonins. The

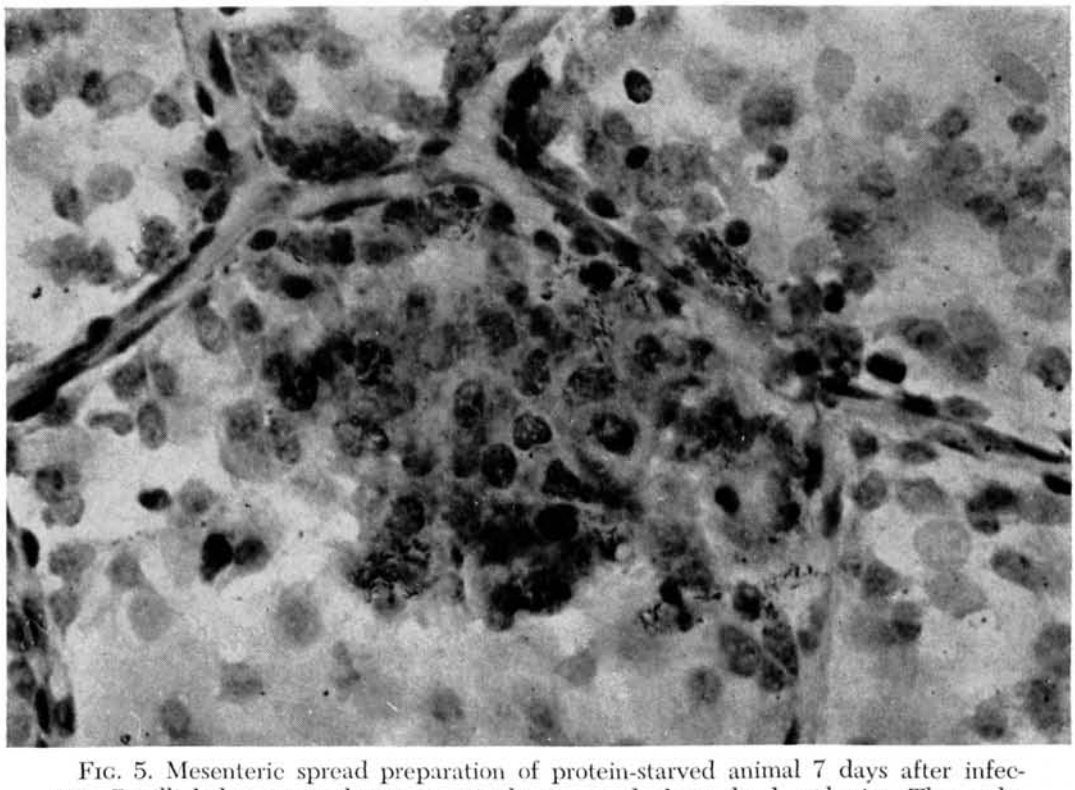

FIG. 5. Mesenteric spread preparation of protein-starved animal 7 days after infection. Bacilli laden macrophages are singly scattered along the lymphatics. The endothelial wall is already damaged and endothelial cells are found containing bacilli. Ziehl-Neelsen stain, magnification, 400X.

lack of similar debilitation of phagocytic activity in the present experiment lends support to the concept, stated above, that in cellular immunity, the defense mechanism resides in the intracellular host cell/pathogen interaction; failure to overcome a pathogen is most likely dependent on failure of adequate intracellular digestion of the pathogen rather than on inadequate phagocytosis.

The tissue sections of the viscera and bone marrow of control and immunized animals did not show specific lesions at this early stage of intraperitoneal infection. There were only small foci of nonspecific chronic inflammatory cell infiltration in the liver, hyperplasia of splenic lymphoid follicles and slight myeloid hyperplasia of the bone marrow. The immunized animals, in addition, showed prominent Kupffer cells and alveolar macrophages. The protein-starved animals showed earlier bacillary dissemination to the viscera than did the other groups. However, owing to the lack of reactive cells, the bacilli in the viscera did not provoke granulomatous inflammation. The bacilli-laden macrophages did not show an accumulation of surrounding epithelioid cells to form granulomas. The spleens of the protein-starved animals were markedly atrophic and were depleted of lymphoid elements. The bone marrows showed marked hypocellularity.

Electron microscopic examination. Tissue sections were not practical for electron microscopic study of the early stage of cell-pathogen interaction. Parasitized cells were too few in early infection to locate even with multiple sections from intravenously infected animals. Therefore, most of the observations on early cell-pathogen interactions were obtained from the peritoneal cell exudate model. After intraperitoneal infection the peritoneal macrophages were readily collected and the pellets contained numerous parasitized cells. For later stages of cell-pathogen interaction (four weeks and later) observa-

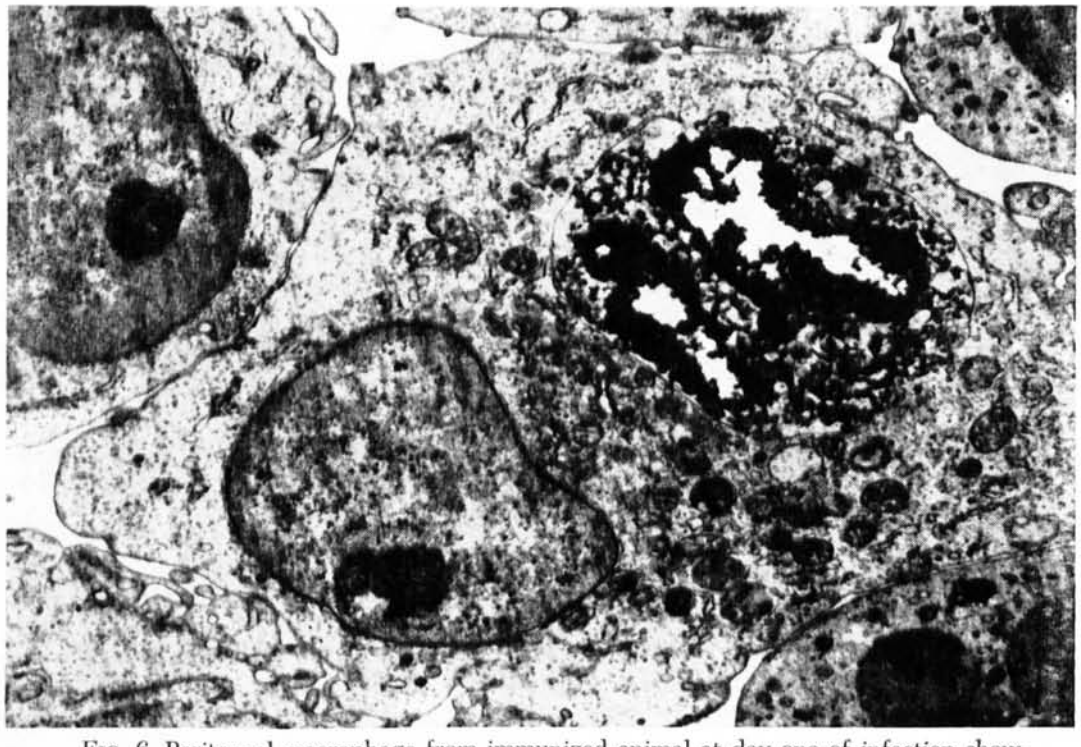

FIG. 6. Peritoneal macrophage from immunized animal at day one of infection showing phagocytosis of degenerated polymorphonuclear leucocyte. Magnification 12,000X.

tion of tissue sections (liver and spleen) of intravenously infected animals are also included.

During the course of infection the striking and interesting observations were the dynamic changes of the macrophages. There was a progressively increasing development of large, mature macrophages. Intracellular modulation consisted of hyperplasia of Golgi complexes with increasing numbers of dense cytoplasmic granules (lysosomes) in and/or around the phagocytic vacuoles. The ultrastructural morphology of peritoneal exudate cells of the control and immunized animals reflected the differences in the rates of such changes in the macrophages responding to the infection. The cellular and intracellular responses of the immunized animals were earlier and more rapid than the controls. It also appeared that there was greater cellular response and cellular activity in the immunized animals as reflected by more prominent Golgi complexes and more lysosomes. Also, at day 1 of the infection, there was more phagocytosis of polymorphonuclear leucocytes (many of which contained acid-fast bacilli) by macrophages in the immunized animals (Fig. 6). The cell granules (containing lysosomal enzymes) and bacilli from the degranulating and disintegrating leucocytes were incorporated into the cytoplasm of the macrophages. Bacilli that were more large mature macrophages in ly were found singly or in groups of two to three in membrane-bounded phagocytic vacuoles. At day 7 of the infection there were more large mature macrophages immunized animals than in the nonimmunized controls. These mature macrophages had hyperplastic Golgi complexes consisting of more than three, and sometimes as many as six bundles of flattened lamellar membranes and numerous Golgi vesicles. Their lysosomes usually varied in size, shape, and electron-density, these variations representing active stages of development. These cells contrasted strikingly with the majority of day 1 macrophages. The latter had comparatively inconspicuous, small Golgi complexes and a few small or medium sized lysosomes. A majority of the lysosomes were spherical or oval with a smooth limiting unit membrane and a cen-

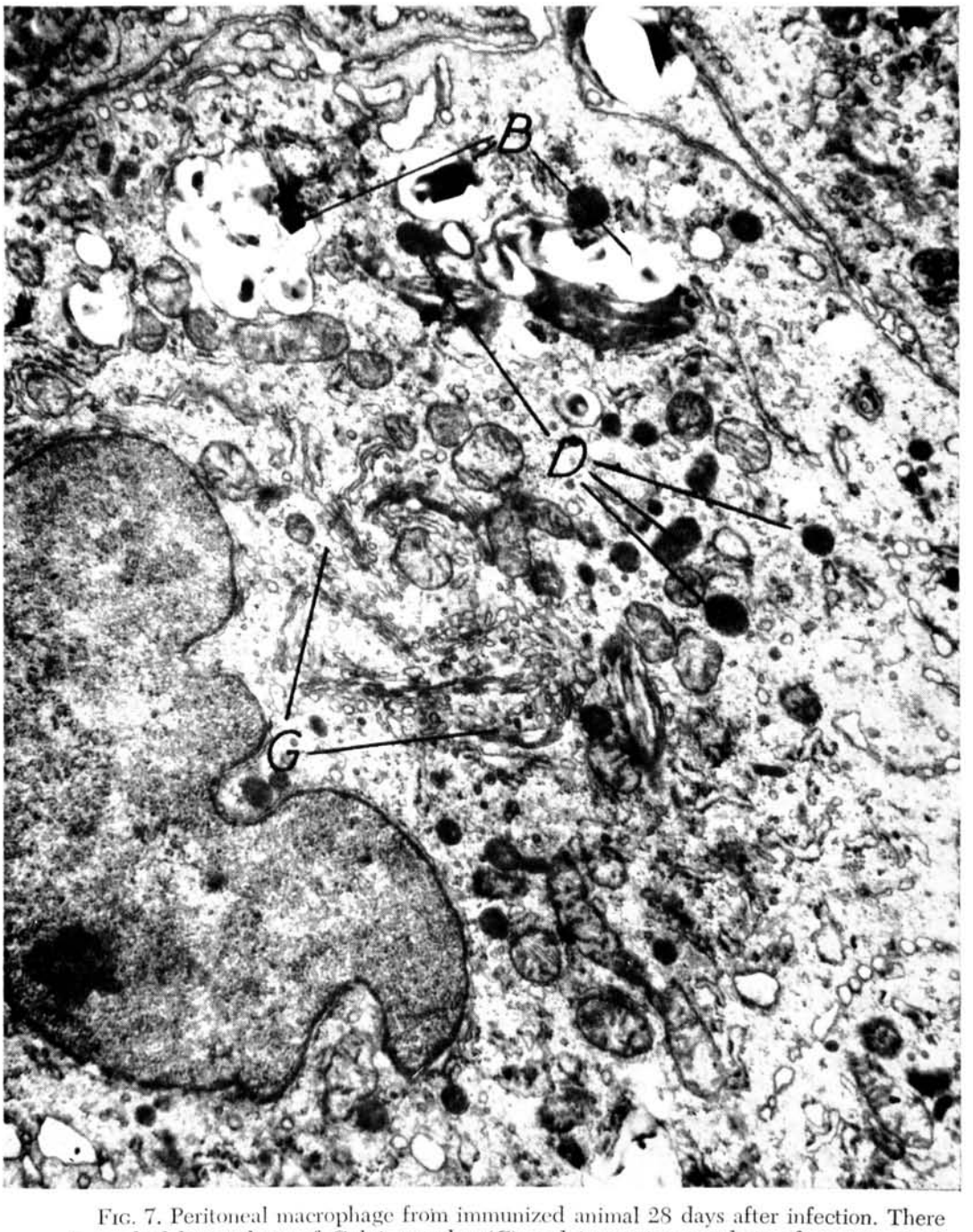

FIG. 7. Peritoneal macrophage from immunized animal 28 days after infection. There is marked hyperplasia of Golgi complex **(G)** and increasing numbers of various sized dense bodies **(D)** around the area of Golgi complexes. Other dense bodies are fusing with the phagocytic vacuoles that contain bacilli **(B).** Magnification, 32,000X.

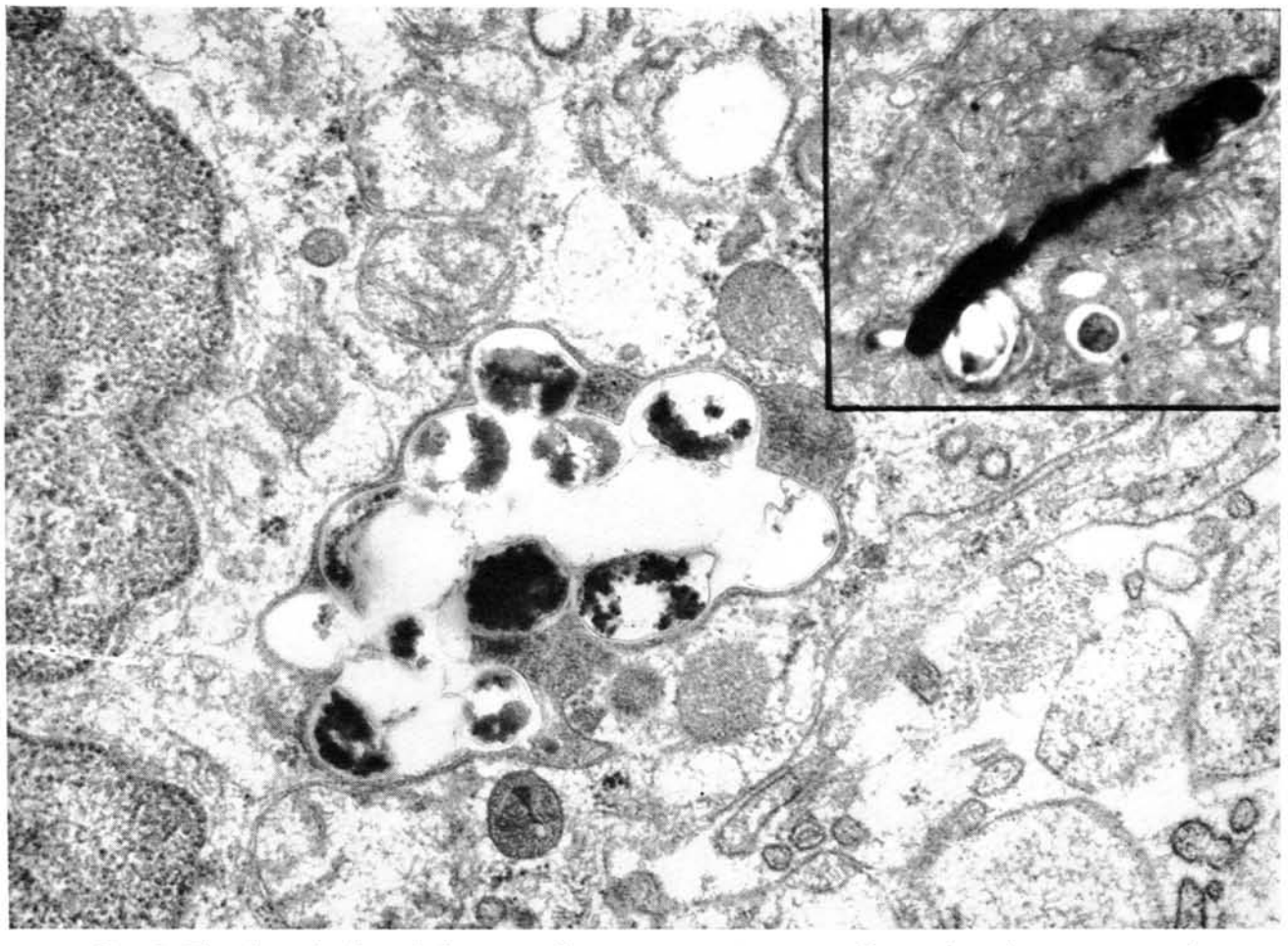

FIG. 8. The dense bodies of the macrophage are seen incorporated into the phagocytic vacuoles. Magnification, 56,000X. *Right upper corner:* A bacillus, longitudinal section, in phagocytic vacuole is being damaged in the middle portion by the presence of dense bodies. Magnification, 28,000X.

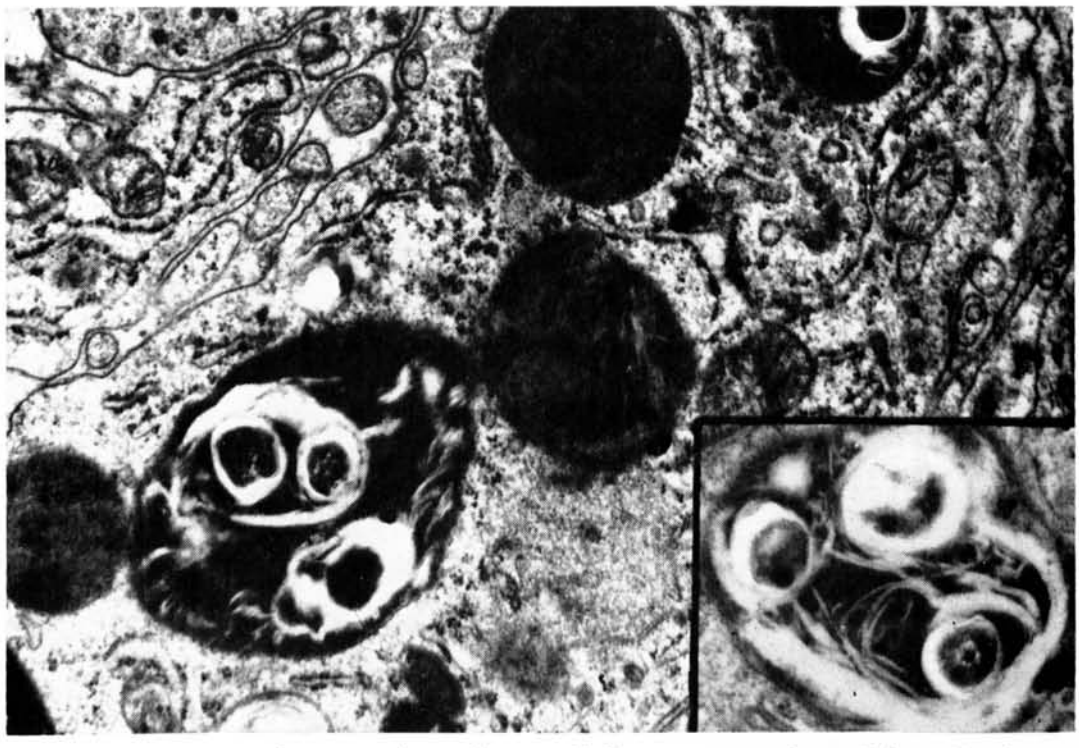

FIG. 9. Portion of a macrophage showing fibillar material peeling off from the degenerated bacilli in the phagocytic vacuoles. Magnification, 48,000X.

tral homogenous dense matrix of uniform density.

By the 15th day, in control and immunized infected animals, most of the cells were large macrophages. The cells from both control and immunized animals showed an increase of lysosomes in or around the phagosomes containing bacilli. However, even at four weeks of infection it was still obvious that in the immunized group there were more lysosomes at various stages of development around the markedly hypertrophied Golgi complexes than in the control animals (Fig. 7). At later stages of infection, after 28 days, the differences became less obvious between the two groups. The majority of lysosomes in infected macrophages showed increased density. Some of the dense bodies were so prominent as to be evident even by light microscopy of the Epon embedded sections (about one micron in thickness) stained with toluidine blue and carbol fuchsin. Different stages in the incorporation of lysosome into phagocytic vacuoles were still evident (Fig. 8). On close contact with the phagocytic vacuoles (phagosome) the lysosomal membrane fused with the membrane of the phagocytic vacuole and eventually the content of the lysosome was discharged into vacuoles containing phagocytosed bacilli, thus forming phagolysosomes. In the dense phagolysosomes there were usually fibrillar structures (Fig. 9) peeling off from the bacilli and many of these bacilli were no longer solidly homogeneous. This change probably indicates degenerative changes in the bacilli ([28]). EM acid-phosphatase stained sections revealed that these peri-bacillary dense bodies (phagolysosomes) were positive for acid-phosphatase (Fig. 10). Cell organelles such as mitochondria, endoplasmic reticulum and nuclei gave no evidence of degeneration or damage even when the cells were heavily infected.

These intracellular reactions, though strong and prominent, were apparently not effective enough to degrade all phagocytosed bacilli. There were undamaged bacilli, which appeared to be well protected by thick "wax capsules." Increasing numbers of

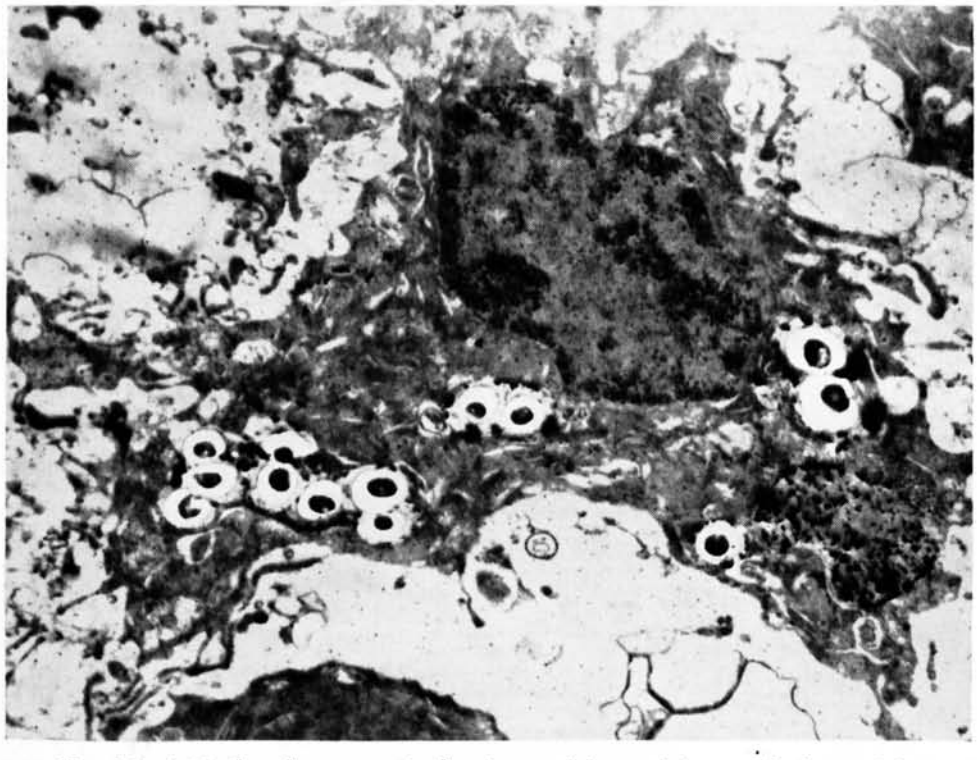

FIG. 10. Acid phosphatase stain showing positive activity as black precipitate in the peribacillary dense bodies and in phagocytic vacuoles. Magnification, 12,000X.

solidly homogeneous bacilli, "degenerated" bacilli, bacillary debris and fibrillar material gradually filled the phagocytic vacuoles of the late stage macrophages. In these cells the cytoplasmic dense bodies (lysosomes) were depleted and the cell organelles tended to disappear.

Macrophages from the protein-starved animals presented marked abnormalities, after six weeks on the protein-free diet, even in the absence of infection. These presented primarily as the presence of a considerable number of myelin figures and atypical, inclusion bodies (Fig. 11). The myelin figures consisted of multi-layered, concentric membranes surrounding irregularly shaped dense central granules. These changes are similar to those produced by Triparanol in the cytoplasm of hepatocytes and pancreatic acinar cells [18] as a result of focal cytoplasmic sequestration due to interference with lipid metabolism. The inclusion bodies were usually represented as oval or elongated vesicles of about 1,000 Å in diameter, having a single unit membrane inclosing segmented dense matrix substance. These changes probably are indicative of some defect in protein metabolism related to protein deficiency. Spontaneous degranulation and lysis of polymorphonuclear leucocytes was evident, without any evidence of phagocytosed inclusions, in protein deprived cells even in noninfected animals. During infection the protein-starved macrophages showed markedly less formation of lysosomes and less fibrillar material from bacillary degeneration was found in the phagocytic vacuoles. The phagocytes were smaller and were more readily damaged during their preparation for electron microscopic examination. Swelling of mitochondria was invariably present. The phagocytic vacuoles were usually large and the electron density of their fluid content was less than that of the rest of the cytoplasm. This probably indicates that there was increased hydration of cell organelles during infection.

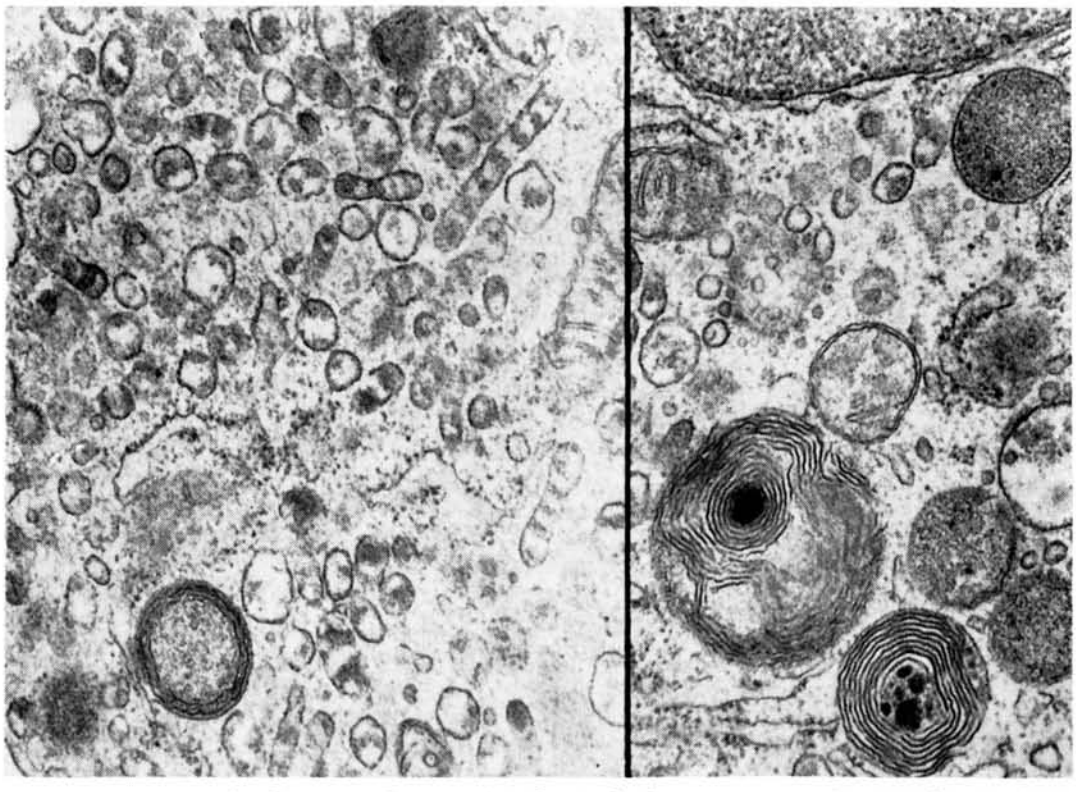

FIG. 11. Myelin figures and atypical inclusion bodies in peritoneal macrophages of protein-starved animals. Magnification, 36,000X.

DISCUSSION

The effective defense mechanism in mycobacterial and other granulomatous infections consists in phagocytosis and intracellular digestion of the pathogen by macrophages. This is basically a phenomenon of native or inherent immunity. Its enhancement is a feature of acquired cellular immunity and seems to be largely independent of humoral antibody development or of any enhanced activity involving other inflammatory cells ([6]). Evidence is accumulating that acquired cellular immunity plays a major role in host defense against infections such as tuberculosis ([21]), leprosy ([32]), brucellosis ([7, 25]), pasteurellosis ([1]), and mycotic infection ([14, 30]). The mechanism of such enhancement is poorly understood.

Mycobacterium lepraemurium infection of the mouse peritoneal cavity presents opportunity for the study of cell-pathogen relationships in early stages before and during the development of cellular immunity and also provides opportunity for contrasting these early cellular and intracellular reactions in immunized and in protein-starved animals. Sequential morphologic study of the peritoneal macrophages and of omental and mesenteric spreads in the early phase of the infection showed the early course of leproma formation following initial phagocytosis of the acid-fast organisms by both macrophages and polymorphonuclear leucocytes. The ineffectiveness of the polymorphonuclear leucocytic response is demonstrated by their lack of participation in the subsequent course of the infection as well as by the fact that within the first few days of the infection the polymorphonuclear leucocytes themselves, together with their modest content of bacilli, were in turn phagocytosed by the macrophages which thenceforth carried the burden of defense. Electron microscopy confirmed this observation. Further development of the leproma was continued by the aggregation of macrophages, and macrophages transformed

to epithelioid cells, around foci of bacilli and macrophages containing phagocytosed bacilli. As contrasted with the course in protein-starved animals, this cell reaction was able to delay dissemination of the pathogen for about three weeks. The immunized animals gave the most effective inflammatory performance, as demonstrated by survival comparison studies in which their more rapid mobilization of defense mechanisms provided a significant lag in dissemination of the mycobacteria; adequate to provide significantly longer survival but not to prevent eventual demise from the infection.

The intracellular dense bodies were shown by electron-microscopic studies to have a distinct relationship to the phagocytosis of the pathogen concerned. They were incorporated into phagocytic vacuoles, they were more prominent in immunized animals, they increased in numbers during the course of the infection, they were diminished in debilitation and here they failed to increase effectively in number in response to the infection in contrast to their response in control and immunized animals. The dense bodies also reflect their content of enzymes in their inclusion of acid-phosphatase.

Morphologic fusion of dense bodies with phagocytic vacuoles has been observed by many investigators ([15, 17, 20, 24, 34, 43]). It seems generally accepted that a phagosome (phagocytic vacuole) is transformed into a phagolysosome by fusion of acid hydrolase containing granules (lysosome) with phagocytic vacuoles. In the present experiments, due to the chronic nature of the cell-pathogen interaction, several kinds of cytoplasmic dense granules (dense bodies) were observed in the macrophages during the course of infection. They apparently represent different stages of development during the interaction between the lysosomes and phagocytized bacilli. The small granules with homogeneous, less electron-dense content, that usually appeared around Golgi complexes (Fig. 7) were most likely the nascent or primitive developing stages of lysosomes which later developed into full-size lysosomes, presumably by accumulating newly synthesized acid hydrolases. These large lysosomes were seen incorporated into the phagocytic vacuoles. Other very dense cytoplasmic granules, could represent condensed digestive vacuoles. A constant pouring out of newly formed lysosome into the vacuoles (phagosome) in the presence of long-standing intracellular stimulation could reasonably be regarded as causing the formation of "very" dense bodies. They were the ones that also had many associated degenerated bacilli and fibrillar material. The dense matrix is certainly a mixture of bacillary metabolites, mycobacterial capsular lipid or wax, and cellular enzymes and even some nonspecific phagocytized materials as, for example, degenerated polymorphonuclear leucocytes. A recirculation or reutilization of the dense granules in the cells, observed by Gordon ([9]), may also occur in these chronic intracellular cell-pathogen interactions.

SUMMARY

Murine leprosy is a useful experimental disease model for study of chronic granulomatous infection and was used to explore the long-termed intracellular parasite-host cell interactions.

The morphologic differences of macrophages in the early stages of development of immunity during the course of infection in immunized, protein-starved and control animals were compared by light and electron microscopic studies. The prolonging of the survival time in immunized animals was associated with quantitative and qualitative enhancement of cellular (macrophage) responses to infections. The macrophages of immunized animals were prominent in formation of granulomas at the site of infection. Ultrastructural examination of these cells indicated rapid, marked increase of lysosomes which then incorporated into phagocytic vacuoles. The protein-starved animals mobilized far less peritoneal macrophage defense against early dissemination of the pathogen. The macrophages of protein-starved animals frequently showed various kinds of focal cytoplasmic degenerative changes and sometimes atypical inclusions. The formations of lysosome was markedly suppressed.

RESUMEN

La lepra murina es un modelo de enfermedad experimental útil para el estudio de la infección cronica granulomatosa y se usó para explorar la parasitación intracelular de larga duración y la interacción celular del huesped.

Las diferencias morfológicas de los macrófagos en las primeras etapas del proceso inmunitario, durante el curso de la infección en inmunizados animales dejados sin proteínas y animales de control se compararon mediante estudios de luz y electron-microscópicos. La prolongación del tiempo de sobrevida en animales inmunizados se asoció con aumentos cualitativos y cuantitativos de la respuesta celular (macrófagos) a las infecciones. Los macrófagos de animales inmunizados fueron prominentes en la formación de granulomas en el sitio de la infección. El examen ultraestructural de estas células indicó un rápido, marcado aumento de los lysosomas los que se incorporaban en las vacuolas fagocíticas. Los animales desprovistos de proteínas en su dieta mobilizaban mucho menos macrófagos peritoneales en su defensa contra la diseminación precoz de los patógenos. Los macrófagos de las animales carentes de proteínas en su dieta frecuentemente mostraban varias clases de cambios focales citoplasmáticos degenerativos y algunas veces inclusiones atípicas. La formación de lysosomas fue marcadamente suprimida.

RÉSUMÉ

La lèpre murine est un modèle expérimental de la maladie, qui est utile pour l'étude de l'infection granulomateuse chronique, et a été utilisée pour explorer les réactions intra-cellulaires reciproques et à long terme entre l'hôte et le parasite.

Par des études de microscopie optique et de microscopie électronique, on a comparé les différences morphologiques des macrophages au cours des stades précoces du développement de l'immunité durant l'infection, chez les animaux immunisés, chez des animaux soumis à un régime de carence en protéines, et chez des animaux témoins. La prolongation du temps de survie chez les animaux immunisés était associée avec une stimulation quantitative et qualitative des réactions cellulaires (macrophagiques) à l'infection. Les macrophages des animaux immunisés se distinguaient par la formation de granulomes à l'endroit de l'infection. L'examen de l'ultra-structure de ces cellules a montré une augmentation rapide et notable des lysosomes, qui étaient ensuite incorporés dans les vacuoles de phagocytose. Les animaux soumis à un régime de carence en protéines ont recouru à une beaucoup plus faible défense macrophagique péritonéale, pour lutter contre la dissémination précoce de l'agent pathogène. Les macrophages de ces animaux soumis à un régime de carence en protéines ont fréquemment montré divers aspects de modifications dégénératives cytoplasmiques en foyers, et parfois des inclusions atypiques. La formation des lysosomes était supprimée.

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