In Vitro Behavior of Macrophages from Healthy Persons Against *M. Leprae* and Other Mycobacteria[1]

J. Delville[2,3]

Beiguelman ([2]) has suggested that the dimorphic reaction in the two polar types of leprosy may be an inherited trait. Beiguelman and Quagliato ([4]) noted in biopsies of leprosy patients that the macrophages of some individuals are able to digest *M. leprae*, whereas those of other individuals are unable to do so. Beiguelman and Barbieri ([3]) and Beiguelman ([5]) related the dimorphic reaction in the two polar types of leprosy and observed a correlation with the Mitsuda reaction. Whereas macrophages from cases affected with the tuberculoid type can completely digest ingested bacilli, macrophages from the lepromatous type are unable to do so, and change into leprosy cells containing numerous bacilli in their cytoplasm and fat droplets stainable with Sudan black. The absence of lysis of bacilli in the macrophages from lepromatous patients is specific for *M. leprae* if intact bacilli are inoculated, and their macrophages are able to lyse *M. lepraemurium* and *M. tuberculosis*.

The dimorphic behavior of macrophages can be observed *in vitro* and *in vivo*. Beiguelman suggested considering the *in vitro* test as evidence of an hereditary trait. Barbieri and Correa ([1]) and Beiguelman ([5]) suggested use of the *in vitro* behavior of macrophages toward *M. leprae* as a prognostic test in leprosy instead of the Mitsuda reaction.

Such a dimorphism has previously been observed by Hanks ([10]) in tissue cultures of tuberculoid and lepromatous lesions. Fibroblasts from tuberculoid lesions were able to destroy *M. leprae*, whereas those originating from lepromatous lesions were unable to do so, and degenerated with liberation of intact bacilli. According to Hadler ([8]) the cells described by Hanks as fibroblasts provided with great phagocytic and atrocytic activity, are fusiform macrophages. If the behavior of macrophages against *M. leprae* in the two polar types of leprosy is determined by genetic factors, it should be possible to note the same dimorphism in healthy persons without contact with leprosy. This was the first objective of our research.

Beiguelman ([6]) has pointed out the interest of carrying on such research in healthy persons. He emphasizes the usefulness of enzymatic research for explanation of the lysis of *M. leprae*, and stresses the observation made by Hadler that the macrophages of guinea-pigs and rabbits, which are able to lyse *M. leprae* and *M. lepraemurium*, have also a high phosphatase (alkaline and acid) and lipase activity, whereas rat macrophages, which are unable to lyse those bacilli, have only weak phosphatase and lipase activity.

MATERIALS AND METHODS

HUMAN MACROPHAGE CULTURES

Our macrophage cultures are developed from peripheral blood of healthy blood donors[4]. After blood for transfusion has been collected and the plastic tube of the bag has been cut aseptically, about 20 ml. of blood are collected in a sterile screw-capped bottle containing 2 ml. Basal Medium Eagle-Diploid Gibco (BME) with 2 mgm. heparin (Roche) per ml.

Sedimentation of the red cells is accelerated by placing the flask at an angle of 45°. The success of the monocyte cultures depends on the rate of sedimentation. The

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[2] J. Delville, M.D., Chargé de cours associé, École de Santé Publique, Université Catholique de Louvain, 4 av. Chapelle aux Champs, 1200 Bruxelles, Belgium.

[3] With the technical assistance of A. Bouckaert and Ch. Lagasse de Locht.

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best results are obtained when a sufficient volume of plasma (3 to 6 ml. or even more), with a large number of white cells, is collected after 30 to 45 minutes. The white cells sometimes sediment at the same rate as the red cells, resulting in supernatant plasma containing too few cells, especially lymphocytes. In such cases, even the buffy coat technique does not give good results. At first phytohemagglutinin was used to accelerate the sedimentation, but this also induces some packing of the white cells and the cultures are less constant and uniform than with spontaneous sedimentation. Polyvinylpyrolidone (PVP) also was used in an attempt to accelerate the sedimentation rate. This yields a greater volume of white cells, but sometimes the growth is not satisfactory.

The plasma after sedimentation is collected in a siliconized tube and diluted with one or two volumes of BME. The mixture is centrifuged at 800 rpm for five to seven minutes, in order to obtain sedimentation of the white cells with a minimum of blood platelets. The supernatant plasma is collected in another tube and centrifuged at high speed (3500 rpm) for at least five minutes in order to get a clear plasma, which is then used in the culture medium. We always use autologous plasma in order to avoid introducing extraneous factors.

The sediment of white cells is resuspended in 2 ml. BME and homogenized. The cells are counted immediately in a counting chamber. At least two million white cells are needed to furnish a satisfactory monolayer type culture, entirely covering the cover-slip of a Leighton tube. The white cell suspension is at once adjusted to an appropriate volume, adding BME and autologous plasma to get a final concentration of 20 to 25 per cent plasma and two million cells per ml. This suspension is then dispensed immediately in Leighton tubes provided with 10 mm. x 40 mm. cover-slips to fit exactly with the flat surface of the tube. The cover-slip is previously fixed to the flat surface of the tube with a 0.5 per cent Noble agar solution in BME introduced by capillarity and coagulated by refrigeration.

The culture tubes, plugged with rubber stoppers, are incubated at 36°-37°. If necessary, the sedimentation of the heparinized blood may be accelerated by spinning them a few minutes at 800 rpm, to obtain a leucocyte layer or buffy coat at the interface between the plasma and the red cells. In this case, however, many red cells are collected together with the white cells. The buffy coat is resuspended, homogenized and dispensed in the same manner as previously described.

The cultures obtained with this technique are, however, less homogeneous. After a few hours, only monocytes adhere to the glass. The non-fixed cells can be eliminated by renewing the culture medium after gentle shaking. It is more advantageous, however, to renew the culture medium only 24 to 48 hours later or even partly to reuse the old medium after centrifugation. The initiation of the culture of monocytes and their transformation into macrophages seem to be favored by the presence of the other blood cells or by some substances liberated from them.

Afterward renewing the culture medium depends on its acidification. Bacteria are normally inoculated after the second or third renewing of the culture medium and the concentration of the plasma is then reduced at 10 per cent. Our technique of monocyte culture from peripheral blood, with monocytes as the only blood cells to fix to the glass, enables us regularly to get macrophage cultures of the monolayer type, sufficient in quality and quantity to permit observation of the evolution of the cells and their action upon the ingested bacilli for a sufficient length of time.

Our cultures are usually maintained alive for three to four weeks, and sometimes more, the longest survival time hitherto observed being seven weeks. This is among the longest survival times recorded in the literature for human macrophages. The macrophages do not seem to multiply *in vitro*. What we get thus is not a cell culture, but only surviving cells. This is in general agreement with similar observations made for mammalian macrophages. Even, after treatment with phytohemagglutinin and colchicine, we were not able to observe mitosis in our macrophage cultures.

:r some time, which differs from one e to another, but which is generally en three and six or seven days, mono- are transformed into macrophages. A ıultinucleated giant cells are already 'ed at this stage. The cultures are noculated with a homogeneous sus- ın of *M. leprae* or other mycobac-

:EPARATION OF BACILLARY SUSPENSIONS

leprosy material is supplied by the ı leprosy hospital, Equator Prov- Democratic Republic of the Congo[5]. nas removed aseptically, excluding taneous epithelium in order to min- the risk of contamination, are ined in BME medium with antibiot- enicillin and streptomycin or penicil- ne, or chloramphenicol, 100 units or gm per ml). They are shipped by Since lepromas are sometimes con- ted with molds or yeasts, nystatin or ne may also be added to the medi- Those antibiotics are washed out liately after arrival of the lepromas.

:omas are separated from the re- g epithelium and fat, washed in vithout antibiotics, then cut in small nts, ground in a tissue grinder, and ded in BME medium with or with- nicillin (100 units/ml.). Coarse par- are eliminated by sedimentation or ration on a five micron-membrane This technique, however, involving a rable loss of bacilli, cannot be gen- l, considering the difficulties in ob- leprous material in sufficient quan- arge masses of bacilli can also be l into small fragments of five to ten by repeated grinding, but with the altering the viability of the bacilli.

neration of bacilli is carried out on a xed and Ziehl-Neelsen-stained After shaking the bacillary suspen- known volume is spread out on a over a calibrated area (about m). The bacilli of five vertical ar- rays (about 400 microscopic fields), select- ed at random, are numbered, using the oil immersion lens. This somewhat laborious technique gives sufficiently reproducible results. The bacillary suspensions are adjusted at about $3x10^6$ bacilli per ml. and 0.1 ml. is inoculated in each culture tube. This yields an average of three bacilli per cell in a tube containing 10^5 cells.

Other mycobacteria, such as *M. tuberculosis, M. pikine, M. marianum* [6], *and M. scotochromogenes*, are cultivated in Dubos medium and diluted to the appropriate dilution in BME. The Dubos medium provides a homogeneous culture and small isolated bacilli. In our initial research, when culture tubes in sufficient number were obtained from a single blood specimen, the tubes were separated into three batches, one being inoculated with heated *M. leprae* (one hour at 100°), a second with unheated *M. leprae,* and a third, kept without inoculation as a control for follow up of the cytologic evolution of the culture in the absence of bacilli. Afterward we left out the heated *M. leprae,* which do not reproduce what happens *in vivo,* and inoculated only unheated *M. leprae* as recently isolated as possible and other above mentioned living mycobacteria. The effect of cortisone was also observed in a few inoculated and uninoculated macrophage cultures.

Usually, 24 hours after inoculation of the bacilli, a first cover-slip was fixed with methanol and stained by the Ziehl-Neelsen method. At the same time the culture medium of the tubes is renewed and the remaining bacilli in the old medium are numbered in order to evaluate approximately the number of ingested bacilli. Cover-slips are then stained every two to three or four days, or even at longer intervals, according to the number of culture tubes, in order to observe the culture for a sufficient period of time. The culture medium is renewed two or three times a week, in order to maintain the proper pH. The old medium is stored in a sterile bottle and,

· thanks to Dr. A. Renders, former medical of the Iyonda leprosy hospital and to Mr. s for their valuable help in forwarding ıaterial.

[6] *M. marianum* and *M. pikine* strains were kindly supplied by Mrs. Fauchet from the "Institut Mérieux, Lyon" and we give her our grateful thanks.

at the end of the experiment, the bacilli are enumerated in order to evaluate the number of undigested bacilli and study their morphology.

RESULTS. *IN VITRO* DEVELOPMENT OF MACROPHAGES

The macrophages, developed from the monocytes of human peripheral blood, are fully comparable to those obtained from guinea-pig or rabbit peritoneal exudates. The cells are somewhat pleomorphic and vary from one culture to another, especially with respect to dimensions, metabolic activity, evolution and survival time, which rarely exceeds six or seven weeks. Yet at that time only a small proportion of the cells survive, especially multinucleated giant cells.

A few hours after initiation of the culture, the monocytes are fixed to the glass and show a triangular or fusiform shape. Later the cells grow more or less rapidly, transforming into macrophages with variable morphology. Besides round amoeboid cells, with dense endoplasm containing the nucleus, and a weakly stained hyaline ectoplasm, one can see cells of fibroblastic or epithelioid type, triangular or more or less elongate, sometimes with cytoplasmic extensions of thread-like shape in connection with other cells. In some cultures cells remain small, do not show an evolution toward typical macrophages, and degenerate after a few days. This happens especially with lipemic plasma.

Whereas at the starting point all cells are single-nucleated, after a few days, the time varying from one culture to another, one may observe cells with two or three nuclei and finally multinucleated giant cells with as many as 20 or 30 nuclei. Some multinucleated giant cells are round, with nuclei arranged in a ring surrounding the dense endoplasm or together in the center of the endoplasm. In other giant cells the nuclei are scattered irregularly throughout the cytoplasm, but still for the most part gathered together. These multinucleated giant cells look almost exactly like the Langhans type of foreign-body giant cells.

Giant cells arise normally in all cultures inoculated with bacilli or not, but the moment of their appearance and their numbe vary from one culture to another. In som cultures cells are gathered together, pro ducing true colonies of macrophages an giant cells, exhibiting high activity wit rapid digestion of the ingested bacill Those colonies result from migration of th cells to a given point. The structure of th macrophages, whatever their morpholog round or triangular, is quite uniform. Th nucleus, disposed more or less centrall round or somewhat egg-shaped, sometim double-walled, is uniformly granular contains as a rule one or two conspicuo nucleoli, and is surrounded by a deep stained and vacuolized endoplasm and hyaline ectoplasm without apparent stru ture. In some cultures one may see also few round and regular cells with uniform basophilic cytoplasm more or less slight granular, little or not vacuolized, and co taining one or several nuclei, mostly fo and rarely five. In these cells *M. lepr* remain unaltered for the longest time.

With lipemic plasma the cells devel only after some delay or degenerate qui ly. At first the cells are round, small a granular; if they do not degenerate, th become progressively fusiform and hyali but remain smaller and less amoeboid, w less marked difference between endo- a ectoplasm. Giant cells are then also smal and less amoeboid, but sometimes num ous. When cells from lipemic blood separated into two batches, one being cu vated with autologous lipemic plasma a the other with an homologous isogr but nonlipemic plasma, the first culture r degenerate quickly, whereas the sec may be normal. This demonstrates the r ious effect of lipemic plasma on the ma phages.-Lipemic plasma can be clea from its lipids, partly at least, by filte the chilled plasma on a 0.45-mic membrane filter.

Up to now 359 blood specimens h been cultivated and 232 macrophage tures, originating from 173 persons of l sexes, have been inoculated with myco teria and examined. Two hundred and r teen cultures were inoculated with *M prae* originating from different leproma being inoculated with filtered bacillary

pensions. Thirty-five cultures were inoculated with other mycobacteria, 16 with *M. tuberculosis*, 14 with *M. pikine*, three with *M. marianum* and two with *M. scotochromogenes*. Twenty inoculated cultures did not permit any conclusion, the cells having degenerated prematurely. Fifty-one cultures have either been used for several assays and adjustment of the technique, or have not been inoculated, because of inadequacy of the cultures. Fifty-six cultures have not yet been examined. The study of these cultures enables us to draw some conclusions and to raise new problems which we shall be able to solve later.

CULTURES INOCULATED WITH *M. leprae*

A few hours after inoculation of *M. leprae* many bacilli are already ingested by the macrophages, and after 24 hours, in active cultures, more than 90 per cent of the inoculated bacteria have normally disappeared from the culture medium and have been phagocytized, 20 to 80 per cent of the cells then containing bacilli. Signs of digestion of bacilli may be observed as soon as 24 to 48 hours after their inoculation. This can explain why many cells do not contain bacilli. The complete digestion of the bacilli, however, takes a relatively long time, depending on the activity and vitality of the cells, although the digestion may be stopped by premature alteration of the cells, this being not necessarily in relation with an effect of the bacilli. As previously observed by Hanks (10), a small number of bacilli are well tolerated by the cells and involve little or no reaction from them, whereas numerous bacilli, either isolated or in globi, produce a more rapid reaction of the cells with segregation of the bacilli in digestive vacuoles.

One can notice large masses of bacilli or globi in advanced digestion, whereas isolated bacilli are still perfectly stained, and isolated bacilli remain also for the longest time in the cell culture. When digestion is not complete, intact isolated bacilli may remain in some macrophages, whereas big masses of bacilli or globi are always considerably altered or even completely digested, the cellular culture degenerating prematurely. The presence of *M. leprae* in the cells does not reduce their survival time significantly, compared with the uninoculated reference culture, whereas *M. pikine*, and especially *M. tuberculosis*, induce rapid degeneration of the cells. The digestion becomes obvious with the appearance of granular and fragmented bacilli, progressively losing their staining properties and breaking up into small yellow-brown or black granules, which disappear almost completely, leaving empty digestive vacuoles. Globi and bacillary masses become porous and less dense, fade, and break up into granular masses. Digesting bacilli are located normally in well defined cytoplasmic vacuoles (phagosomes or lysosomes), often near the nucleus. In the giant cells they are located chiefly between the nuclei.

When cells degenerate before the digestion is complete, the bacilli remain inside the cells. If the culture is still active enough, degenerated cells may be ingested by other cells, chiefly giant cells, which can then complete the digestion. In some cases the digestion takes place so rapidly that, even after 24 hours, the inoculated bacilli, having disappeared from the medium, can no longer be found inside the cells. The rate of digestion depends on the number of giant cells. In cultures with many giant cells at the time of inoculation, large masses or globi do not appear.

The cellular reaction against heated bacilli seems less active. Heated bacilli are more slowly digested. Only isolated bacilli, small masses of bacilli or small globi are seen, but never cells stuffed with perfectly stained bacilli, as in cell cultures inoculated with fresh unheated bacilli. In all the macrophage cultures from healthy persons aspects of bacillary digestion were observed. However, marked quantitative differences were noted, as well as differences in the time required for digestion.

One may conclude that among healthy individuals the dimorphism observed by Beiguelman in leprosy patients was not observed.

After a few days, in some macrophage cultures, inoculated with fresh unheated bacilli, one can see some macrophages or

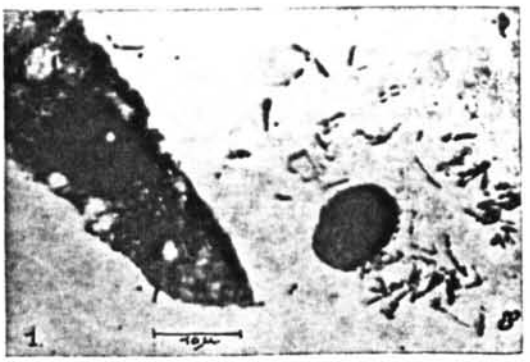

FIG. 1. Ten day old human macrophage culture, inoculated on the fifth day with a suspension of unfiltered *M. leprae*. Numerous bacilli, several uniformly stained, in macrophages.

cells of the epithelioid type containing numerous bacilli. These bacilli are scattered in the cytoplasm, or disposed in a ring around the nucleus, or still gathered together in compact masses or globi reaching a diameter of 20 microns or more, (Figs. 1, 2 and 3). At the same time, bacillary masses or globi are being digested by macrophages (Figs. 4 and 5), and especially by the giant cells, the largest masses often being more rapidly and completely digested than the smaller ones or the isolated bacilli. It seems as though the enzymatic digestive mechanism in the cell is released by a threshold phenomenon. It may happen that a cell contains at the same time digested bacilli and uniformly stained bacilli. All those observations are noted even with an inoculum previously filtered on a five-micron-membrane filter.

When the same inoculum is maintained for a long period at 36°-37° in the same culture medium, but without cells, it does not exhibit globi or large bacillary masses, but shows only isolated bacilli or small masses of less than five microns, which keep their initial shape and staining characteristics. When the inoculated culture of cells consists mainly of giant cells, digestion occurs very soon and cells stuffed with bacilli, or containing big bacillary masses or globi, do not appear. Each time the culture medium is renewed, the old medium is preserved. When all cover-slips have been harvested, all culture media are pooled and

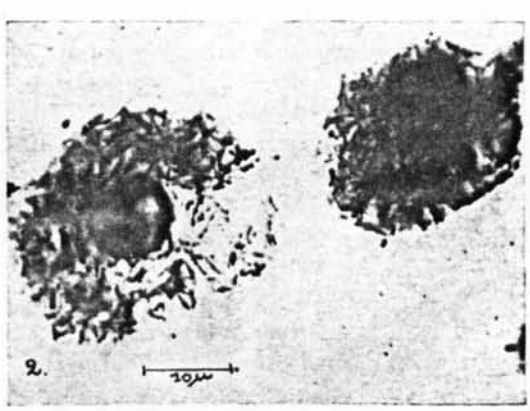

FIG. 2. Thirteen day old human macrophage culture, inoculated on the fifth day with a filtered suspension of *M. leprae*. Macrophages stuffed with bacilli.

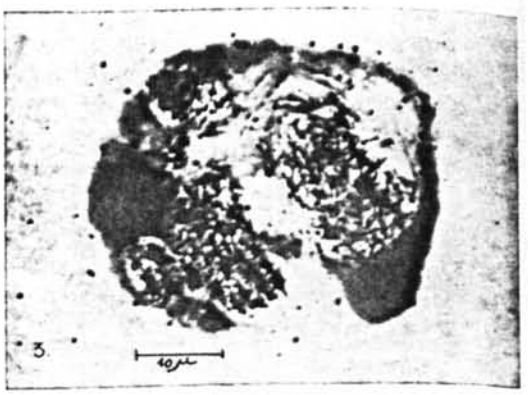

FIG. 3. Same culture as Fig. 2. Bacilli gathered together, and isolated faded bacilli.

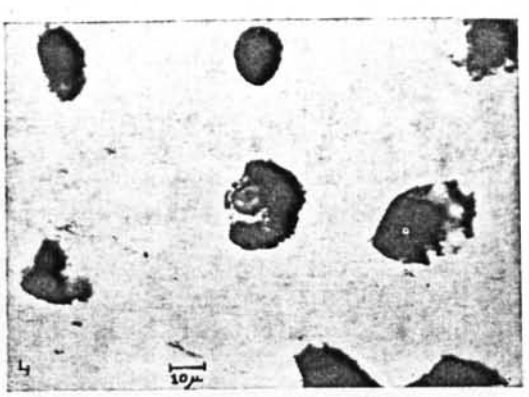

FIG. 4. Same culture as Fig. 2. Large mass of granular bacilli in course of digestion around nucleus.

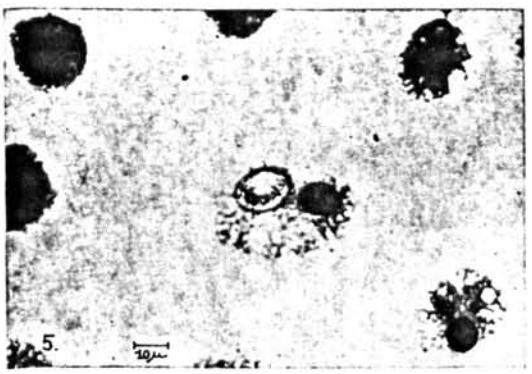

FIG. 5. Same culture as Fig. 2, but 15 days old. Globus in course of digestion by a macrophage.

centrifuged and their sediment is examined. The bacilli in the sediment are more or less numerous, depending on the activity and the survival time of the cell culture. They are generally attached to cellular debris. The sediment also contains degenerated cells with or without altered bacilli. The bacilli in the sediment are for the most part granular and present peculiar staining characteristics. The sediment also contains incompletely digested bacillary masses or granular remnants staining yellow-brown or black. Sometimes the sediment contains uniformly stained bacilli and even, though rather unusually, more bacilli than in the inoculum, whereas in the meantime typical figures of digestion are visible in cells close by, which contain numerous perfectly stained bacilli. In two instances, such bacilli were reinoculated into other macrophage cultures and figures suggesting bacillary multiplication were visible in the vicinity of cells with digestion.

MACROPHAGE CULTURES INOCULATED WITH OTHER MYCOBACTERIA

Thirty-five macrophage cultures were inoculated with mycobacteria other than *M. leprae*, i.e., *M. tuberculosis*, *M. pikine*, *M. marianum*, and *M. scotochromogenes*. In such cultures, 24 hours after the inoculation, almost all the cells contain a few dumpy bacilli. After ingestion some bacilli are rapidly digested, whereas others elongate before multiplying. Three or four days after inoculation, some cells contain many long bacilli (Fig. 6), either isolated on in bundles, whereas in other cells bacilli have disappeared. From then on the number of highly infected cells increases gradually and they detach from their support. Finally most cells are stuffed with bacilli, and the survival time of macrophage cultures inoculated with those mycobacteria, especially with *M. tuberculosis*, is much shorter than that of those inoculated with *M. leprae*. *M. tuberculosis* seems particularly toxic for the macrophages, even in the presence of streptomycin, which does not prevent the intracellular development of the bacilli. These cellular cultures can rarely survive the infection more than 10 to 12 days. Some cells, especially giant cells, with various degrees of alteration, which contain in their cytoplasm a few or no bacilli or granular and fragmented, poorly stained bacillary masses, may still survive for a longer time

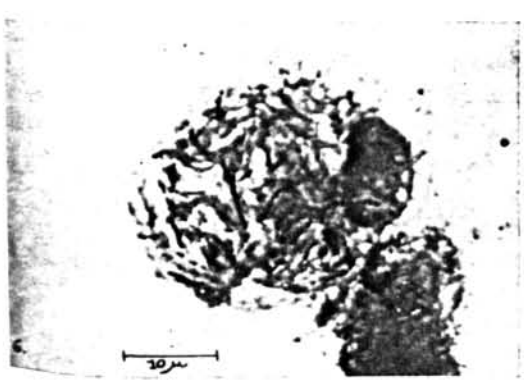

FIG. 6. Eighteen day old human macrophage culture, inoculated on the sixth day with *M. pikine*. Very long bacilli in a macrophage.

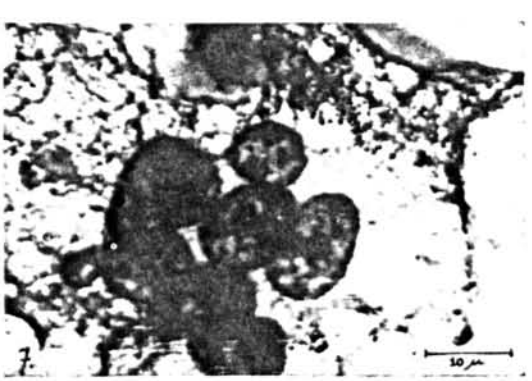

FIG. 7. Same culture as Fig. 6. Giant cell with granular debris of bacilli in course of digestion.

(Fig. 7). In rare instances globus-like bodies are present.

In very active cells, resistant to infection, only granular bacillary masses are present. These have lost their acidfastness and stain yellow-brown or black. Finally, bacillary multiplication occurs in the culture medium outside the cells, whereas during the first days of the infection all bacilli were inside the cells. In *M. tuberculosis* infection, hairy bacillary masses float on the surface of the culture medium. Heavily parasitized cells give rise to microcolonies, which emerge from the cells and continue their development outside. We suppose that the elongation of the bacilli observed in our cultures is due to the presence of penicillin. For this purpose we have inoculated *M. tuberculosis* and *M. pikine* in penicillin-containing Dubos medium and BME.

Although some degree of elongation appears in these media, particularly in BME, it is less marked, however, than in the cells. Inside the cells the development is also faster and more abundant, with threadlike elements. The granular bacilli and others which have lost their acid-fastness, are present also in the vicinity of well stained acid-fast bacilli, sometimes with the appearance of a capsule. All the macrophage cultures inoculated with the different mycobacteria, have, at least at the start and in a few cells, shown figures of bacterial digestion. The cellular cultures, however, are more or less rapidly overwhelmed by the bacterial development and consequently often degenerate prematurely.

EFFECT OF HYDROCORTISONE ON CULTURES INOCULATED WITH *M. leprae*

In eight macrophage cultures, inoculated with *M. leprae*, hydrocortisone was added in a dose of 20 μgm. per ml. of medium.[7] No marked difference was observed with respect to digestion of the bacilli or appearance of cells filled with *M. leprae* or containing bacillary masses or globi in relation to the presence or absence of hydrocortisone.

[7] We express our grateful thanks to Prof. Dr. Fanard, Director of "Organon-Belgique", who kindly supplied us with hydrocortisone (Hydro-Adreson-aquosum).

DETECTION OF LIPIDS AND ACID PHOSPHATASES IN MACROPHAGES

In six macrophage cultures inoculated with *M. leprae*, and in a few uninoculated cultures, we tried to detect the presence of lipids, using the Sudan black and Fettrot techniques. Lipids were detected in both inoculated and uninoculated cultures, and their presence seems to be related to ageing or degeneration of the cells. However, in cultures with lipemic plasma, they were present at the beginning. In seven cultures inoculated with *M. leprae*, and in a few uninoculated cultures, we tried also to detect the presence of acid phosphatases at different stages of development, using the technique of Weissenfels ([12]). Phosphatases were detected in all cultures. Their activity, however, is not the same in all cells. It is most marked in giant cells and in healthy macrophages. Cells with low phosphatase activity and containing numerous bacilli were observed in the vicinity of cells with high phosphatase activity containing fewer bacilli not yet altered.

Hadler ([9]) found a different enzymatic activity in macrophages lysing *M. leprae* or *M. lepraemurium* from that of those which do not lyse these bacteria. Whereas guinea-pig and rabbit macrophages are able to lyse *M. leprae* and *M. lepraemurium* and exhibit a high phosphatase activity, rat macrophages, which do not lyse these bacilli, have only a low phosphatase and lipase activity. It should be worthwhile to investigate the enzymatic activity displayed by cells, respectively from lepromatous patients who do not lyse *M. leprae* and from tuberculoid patients who do lyse the bacilli.

DISCUSSION

We have seen that all our macrophage cultures from healthy persons inoculated with *M. leprae* or other mycobacteria, exhibit a more or less precocious and intense lytic activity against those mycobacteria. This lytic activity is essentially dependent on the quality and survival time of the macrophages, as well as on the time at which giant cells appear. *M. leprae* is particularly well tolerated by the macrophages. These seem not to suffer from any

toxic effect, whereas, after a transitory lysis, they are rapidly overwhelmed by the development of other mycobacteria, especially *M tuberculosis*, and then degenerate. Some of the *M. leprae* suspensions which were inoculated into macrophage cultures, gave rise to the appearance of globi or more or less large bacillary masses and cells filled with bacilli, several of which often grew longer and were uniformly stained, whereas the same suspensions inoculated in the same culture medium, but without cells, never produced the same figures. Consequently the presence of cells is essential to the occurrence of these figures. These observations suggest an intracellular bacillary multiplication or successive phagocytosis.

If successive phagocytosis is the explanation, the ingested bacilli should maintain the morphology of the inoculated bacilli, which should evolve only toward digestion, without elongation or occurrence of uniformly stained bacilli. Moreover, once the medium has been renewed after 24 hours, no bacilli could be ingested without destruction of the cells and release of the bacilli into the medium. In the same way, there should be no difference between heated and unheated bacilli, nor between bacillary suspensions and macrophage cultures from different sources.

The numerical increase of bacilli by successive phagocytosis does not agree with the observation that, most often, more than 90 per cent of the inoculated bacilli have disappeared from the culture medium after 24 hours, at which time the culture medium is renewed. Macrophage cultures, inoculated with heated bacilli, sometimes show small globi and a few cells containing rather numerous bacilli when first examined after 24 or 48 hours. These, however, are never as numerous as in cultures inoculated with unheated bacilli. On the other hand, in macrophage cultures inoculated with unheated bacilli, large globi, bacillary masses or cells filled with bacilli, appear only after a few days and at the earliest after two or three days, at a time when the culture medium has been renewed at least once. The inoculation of a filtered suspension is still more demonstrative. With this procedure only a few bacilli are seen inside the cells after 24 hours. Aspects suggestive of bacillary multiplication appear only after a few days.

Another objection could be that the increase in the number of bacilli in some cells results from phagocytosis of bacilli liberated by prematurely destroyed cells. On the contrary, it is in seemingly active macrophage cultures, without any premature alteration of the cells, that large globi and bacillary masses and cells stuffed with bacilli do occur. Those figures suggesting bacillary multiplication do not occur in poor cultures with early degeneration, which, however, show signs of digestion. Also worth reporting is the observation that *M. leprae* suspensions from the same origin, inoculated in macrophage cultures of different sources, do not necessarily undergo the same evolution.

In some cultures one finds only a small number of bacilli in the macrophages, although they have disappeared from the culture medium, globi or cells filled with bacilli not being observed. This occurs especially in cultures with rapid production of giant cells and rapid digestion. In other cultures globi or bacillary masses and cells filled with bacilli appear more or less rapidly, but digestion always follows. In such cases one might also see uniformly stained bacilli undergoing notable elongation. On the other hand, macrophage cultures originating from the same individual at different moments, but comparable with regard to quality, have the same behavior against *M. leprae* from different sources as far as suspensions are of similar age; globi and cells filled with bacilli are observed, or bacilli disappear more or less rapidly before globi or cells filled with bacilli could appear.

On the basis of those observations we are tempted to admit that globi or bacillary masses and cells filled with bacilli are the expression of bacillary multiplication. This multiplication, of course, is only transient in macrophage cultures from healthy humans, in which the bacillary development induces the production of the enzymes required to destroy those mycobacteria. These enzymatic reactions seem more marked in the

presence of unheated bacilli showing figures of multiplication, than with heated bacilli. The digestion is also more active in cells containing numerous bacilli, especially if these are clustering in globi or bacillary masses, than in cells containing only a few bacilli. If, as our observations suggest, those bacillary pictures in human macrophage cultures correspond with a multiplication of *M. leprae*, this leads to the assumption that the generation time is much shorter than in the mouse footpad, where, as one ought to admit, *M. leprae* is not in its natural biotope and consequently not in optimal conditions for the minimum normal generation time. *M. tuberculosis* also seems to multiply more rapidly in macrophages than in the classic culture media.

In human macrophage cultures, *M. leprae* is at least temporarily in more nearly normal conditions. In the human host *M. leprae* live in lymph nodes, and more precisely in histiocytic cells, As Jadin and Wery (11) have shown, lymph node puncture fluid from leprosy patients may give rise at least to a limited bacillary multiplication. Macrophage cultures derived from blood monocytes, are cells of the same origin. Consequently, one might expect that they could follow the same behavior. The concentration of *M. leprae* in some cells is difficult to explain if no multiplication occurs, more especially as they are comparable with the figures obtained in the same cells inoculated with *M. tuberculosis* and *M. pikine*, which undoubtedly are the result of a bacillary multiplication. Investigation of this phenomenon and of the enzymatic or other relevant reactions, could permit elucidation of some mechanisms in the multiplication of *M. leprae*.

One cannot expect, however, to obtain a continuous culture of *M. leprae* in human macrophages, especially from healthy individuals, because of their lytic activity. First of all we need a cell having no lytic activity against *M. leprae*, which can be maintained alive for a long time without subculture. We have obtained such a cell line, isolated from a Kaposi angiosarcoma, which grew slowly and could be maintained alive for several months without subculture. As we reported at the Rio de Janeiro leprology congress in 1963 (7), those cells inoculated with bacilli obtained from freshly collected leproma, allowed multiplication of *M. leprae*, but a continuous culture could not be maintained. Unfortunately our investigation was stopped prematurely by a technical incident, which involved the loss of our cell line, and up to today we have not had the opportunity to isolate a new strain.

SUMMARY

The purpose of our paper is to relate our research on the *in vitro* behavior of macrophages from healthy persons without contact with leprosy toward *M. leprae* and other mycobacteria. If the dimorphic reaction of macrophages against *M. leprae* in the two polar types of leprosy is an inherited trait, as Beiguelman and Barbieri suggest, then we should be able to detect the same dimorphism in healthy persons. Monocytes from the peripheral blood of healthy donors from a blood bank are cultivated on cover slips in Leighton tubes in BME enriched with autologous plasma. Normally between the third and sixth day monocytes have grown sufficiently and macrophages and even giant cells are present. Mycobacteria are then inoculated, 2 to 3 x 10^5, into each tube.

A homogeneous suspension of *M. leprae* is prepared from lepromas by sedimentation or by filtration through a five-micron-Gelman membrane filter. *M. tuberculosis*, *M. marianum* and *M. pikine* are grown on Dubos medium. About 24 hours after the inoculation a first coverslip is harvested and later another one every three or four days. The cover slips are fixed with methanol and stained by the Ziehl-Neelsen method. At the same time the medium is renewed and afterward again every two to four days, depending on the pH of the medium. The old medium is stored in a sterile bottle and the bacilli are counted at the end of the experiment. A few hours after the inoculation bacilli are already ingested by macrophages and giant cells, and after 24 hours more than 90 per cent of the inoculum normally has disappeared from the culture medium.

The number of cells containing *M. leprae*

varies from 10 to 50 per cent and even more, and the number of bacilli in each infected cell ranges from one to about 10. In some cultures several bacilli have already disappeared 24 hours after inoculation, and fragmented and loosely stained bacilli can be seen inside the cells near uniformly stained bacilli. Later on, after a few days and depending on the freshness of the bacilli and their vitality and the activity of the cells, several cells may contain numerous bacilli dispersed in the cytoplasm or aggregated in globi, the largest from eight to 20 microns in diameter. There seems to be a transient multiplication of *M. leprae*, followed by a digestion which is almost complete if the macrophages remain viable for at least two to three weeks after inoculation. In other macrophage cultures, especially when numerous multinucleated giant cells appear rapidly, globi and cells full of bacilli are not seen, and digestion of bacilli starts immediately. In more than 200 macrophage cultures from healthy persons inoculated with *M. leprae* we have not yet seen any without evidence of digestion if the cells remain viable for a long enough time.

The fate of other mycobacteria, cultivable *in vitro*, such as *M. tuberculosis*, *M. marianum* or *M. pikine*, is quite different. Almost all the cells contain bacilli 24 hours after their inoculation and at that time the shape and length of the bacilli is the same as in the inoculum. In some cells, especially in giant cells, digestion starts quickly, whereas in other cells one notes an elongation of the bacilli followed by multiplication. Some cells stuffed with bacilli, without any evidence of digestion, or even after digestion has begun, degenerate and are the starting point of a microcolony and a culture outside the cells. Bacilli multiply and cells degenerate progressively, macrophages first and giant cells afterward. In most instances, especially with *M. tuberculosis*, almost all the cells degenerate after six to 10 days, when all the cells and even the culture medium are overwhelmed with rapidly multiplying bacilli.

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