MAST CELL RESPONSE IN MURINE LEPROSY [1, 2, 3]

LASZLO KATO, M.D. and BELA GOZSY, Ph.D.

Institute of Microbiology and Hygiene
University of Montreal
Montreal, Canada

The behavior of the capillary endothelium and alterations in the pericapillary space in murine leprosy, have been studied in our laboratories ([2, 3, 5, 6, 7, 8]). We have reported that, in the early stage of the disease, an increased alertness of the capillary endothelium occurs, not only at the site of and around the incipient granuloma but throughout the entire body. Even in histamine- or serotonin-depleted rats, which do not react to intradermal injection of histamine liberators, there was a strong capillary response. When very large necrotic lesions developed, this stimulated readiness of capillary endothelial cells was no longer maintained and, as the disease advanced to a fatal conclusion, the capillary endothelium remained passive to stimulation, and neither India ink nor acid-dye accumulated in and around the lesions ([6]). This characteristic capillary response in murine leprosy remained unexplained at the time, but was investigated further when new technics were developed for exploration of capillary and connective tissue responses to injury ([7]). A "transparent skin technic" ([3]) permitted deeper penetration into the mechanism, demonstrating the mast cell response in the disease, as well as the effect of drugs on the granuloma formation. The results of these investigations will be reported in this communication.

MATERIALS AND METHODS

Male and female albino rats of the Wiersing strain, weighing 50-60 gm., were kept on a standard diet. The bacilli from a three-month-old rat granuloma were separated from the tissue by successive centrifugation, washed, and diluted with saline to 20 times the weight of the granuloma. Ninety rats were inoculated with 0.4 ml. each of the freshly prepared suspension of bacilli, subcutaneously in the center of the abdominal skin. For control 90 rats received 0.4 ml. saline solution. Mast cell response as shown in lesions examined microscopically, was studied at different time intervals after experimental transmission, by means of transparent skin technics ([3]). Capillaries were visualized by intravenous injection of India ink 40 minutes before the animals were killed by decapitation. Gunther's India ink was diluted with saline to contain 3% carbon, with 2% gelatin added. This was heated to 40°C and 0.5 ml./100 gm. body weight was injected into the tail vein.

Drugs were incorporated in the diet, or injected subcutaneously, in the doses indicated in "Results." Eighty rats were divided into four groups of 20. All were infected simultaneously with the inoculation of the previous group; these served as controls for the drug-treated rats. Treatment started on the day of infection, and, except for dihydrostreptomycin, which was injected five times a week, was continued for 80 days. On the 80th day all animals were killed and subjected to autopsy.

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Transparent skin technics.—The evening before the experiments began, the abdominal skin of the animal was shaved, after use of a Neet depilator suspension. Forty minutes after intravenous administration of India ink, the animal was killed by decapitation and stretched on a plate. The skin was cut laterally on one side of the abdomen; this incision was followed by a horizontal cut above the symphysis pubis and an additional horizontal cut at the distal part of the sternum. The skin was then gently removed by hand and stretched over the hollow part of a centrifuge tube stopper (International Equipment Co., Boston. Cat. No. 645, 28 mm. diameter). The glossy connective tissue, with the murine leprosy granuloma in its center, was exposed flatly like a drum, with the hairy dermis stretched over the stopper. A quarter-inch-thick plastic plate (2 × 2 inches), with a hole bored in its middle (28.5 mm.), was then pressed on the skin above the stopper, in such a way as to press the skin between the stopper and the hole of the plastic plate. When unnecessary skin has been cut away with scissors in this procedure, the skin removed is perfectly and flatly stretched.

The skin preparation was stained for one minute with a 0.1% aqueous solution of toluidine blue and 4% formalin and washed with tap water. Excess water was then removed. The stained skin, still attached to the apparatus, was submerged in toluol for 3 hours. Excess toluol was removed and the skin was left on the apparatus overnight to dry. Following dismantling of the stopper and ring, the skin was cut to obtain a ¾ inch square section of the skin. The stained dried skin so obtained is as thin as paper and as transparent as parchment. When mounted on glass slides and fixed with Scotch tape, it can be examined without a cover glass, with the use of transparent light or a contrast phase microscope, ranging in power from small magnification to oil immersion.

The numbers of intact and disrupted mast cells were counted in the transparent skin, after it had been stained with toluidine-blue-formaldehyde. One hundred fields were counted, in a 450-fold magnification of each sample of the abdominal skin removed from the site of infection. The average number of mast cells was calculated from ten rats for each group each time, and plotted in the tables. A mast cell was considered disrupted when more than eight granules were found outside the cell.

The size of murine leprosy granulomas was estimated on the basis of the maximum and minimum diameters of the lesion, as the average area in square mm. of the ten rats killed. This method of expressing the extent of the granuloma gives quantitative information on the size of the lesion. It was recognized that the shape of the subcutaneous granuloma in the abdominal skin differs from that which develops when rats are infected at the scapular region or the dorsum. When rats are inoculated with carefully homogenized washed bacilli, the resultant granuloma creeps flatly in the subcutaneous connective tissue for about 90 days instead of developing into a very large, egg-shaped tumor.

The intensity of carbon accumulation from the India ink injections at the site of infection was estimated as 0, ±, +, ++, +++, and ++++. On each occasion, the number of mast cells, the extent of the granuloma and the intensity of carbon accumulation, were measured on the same group of ten rats.

RESULTS

An early accumulation of mast cells at the site of infection was noted as soon as five days after subcutaneous injection of murine leprosy bacilli into the abdominal skin of rats. In and around the slowly progressing microscopic lesions, the number of mast cells increased rapidly, reaching a peak about 40 days after infection. At this time the massive accumulation of tissue mast cells resembles a mastocytoma, when examined in fields near a microscopic incipient lesion (Figs. 3A, 3B). The mast cells were apparently normal, characterized by metachromatically or ametachromatically (aqueous formalin fixation) stained granules. The cells were normal in size, and the number of

disrupted mast cells was proportionally the same as in the noninfected rats.

With progression of the subcutaneous murine leprosy lesion, profound alterations occurred in the mast cell population at the site of infection and in the surrounding area. The number of intact mast cells suddenly fell far below the normal, and mast cell disruption occurred in extremely high numbers 50 days after infection. Two typical varieties of mast cells appeared at this stage: small, mainly in the pericapillary space, and giant, in groups in the connective tissue located at sites remote from the capillaries. Such giant mast cell herds are illustrated in Figure 3C. Both types of cells retained their normal staining properties. Even at this stage mast cells were seldom found in the microscopic lesion and no mast cell granules were found in the histiocytes of the lesions.

In rats autopsied 50 to 80 days after infection, granuloma formation was recognized as a tissue proliferation extending flatly in the abdominal connective tissue. Its size could be measured easily as a 0.5-2 mm.-thick granuloma of flesh color. Within this period of time, the lesion could still be examined with the transparent skin technic, but not later. Within 50-80 days after transmission, the number of intact mast cells became smaller and smaller. They disappeared completely in the granuloma and underwent destructive changes in the vicinity of the lesions. Those near the histiocyte infiltrations were usually small and for the most part disrupted. Again, no mast cell granules were ingested by histiocytes. On the 80th day mast cells were found only rarely in and around the granuloma (Fig. 3D). Fluctuations of the intact and disrupted mast cells occurring during the 80 days after infection are shown in Figure 1.

On each tenth day, when animals were subjected to autopsy in groups of ten, India ink was injected intravenously 45 minutes before decapitation. Carbon accumulation at the site of infection was estimated at the inner side of the removed abdominal skin. Figure 1 shows that, in the pregranulomatous stage, carbon particles accumulated only weakly in the abdominal skin, at spots infected with the bacilli. However, the intensity of accumulation was very great 45-60 days after infection, mast cell disruption also being maximal, while granuloma formation had progressed. In the transparent skin it was evident microscopically that carbon particles accumulated at the inner surface of the capillary wall and in the capillary endothelial cells. At this time there was a capillary "endothelial activation" ([3]).

The area of the subcutaneous granuloma, measured in mm.^{2}, was considerably reduced when rats were treated for 80-83 days with drugs having an established antileprosy effect in man. Isoniazid in the diet (0.01%) and daily injections of 2 mgm. dihydrostreptomycin showed the highest activity; (p-N,N-dimethyl aminophenyl)-1(p-n-butoxyphenyl)-3 thiourea (Ciba 1906) and 4-4′-diaminodiphenyl-sulfone

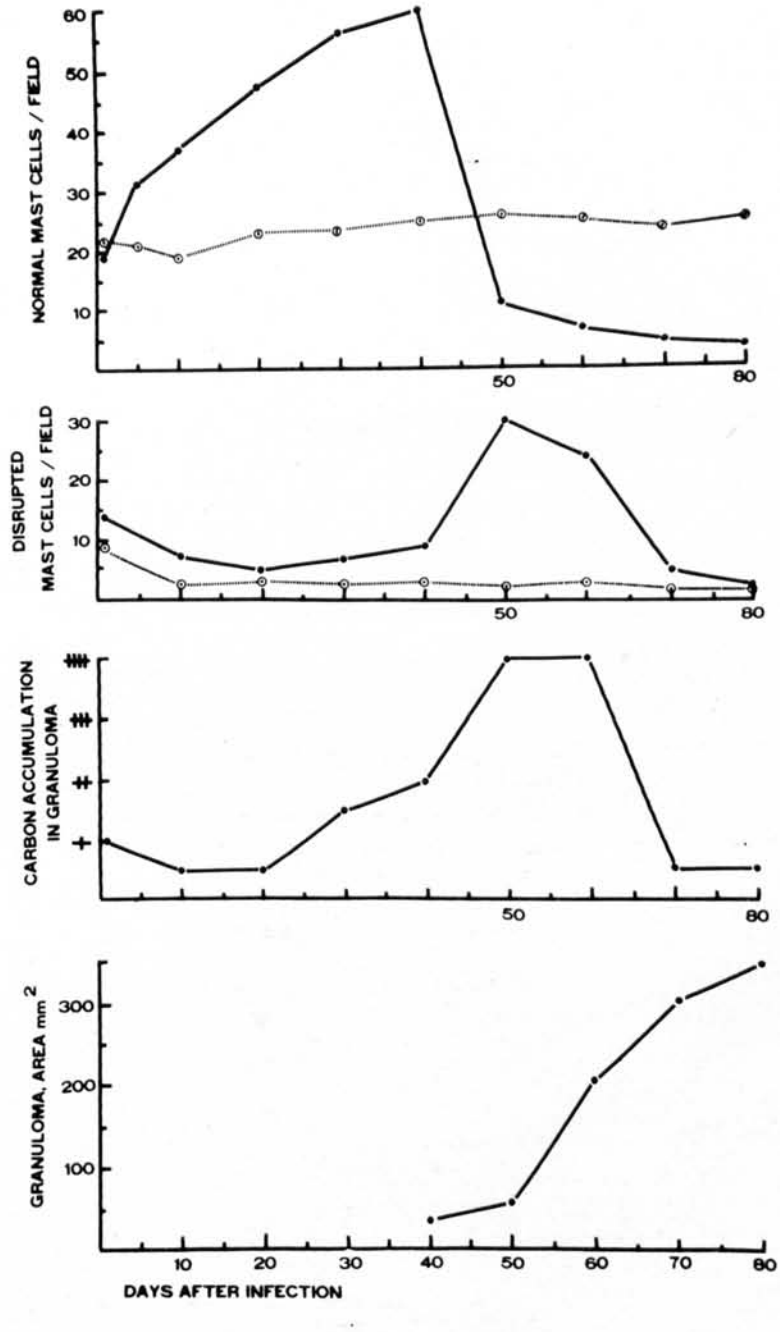

FIG. 1. Average number of intact and disrupted mast cells per field in the abdominal skin of normal rats (⊙·····⊙) and rats bearing subcutaneous murine leprosy granulomas (●——●) at the sites of infection, compared with intensity of carbon accumulation in the granuloma after intravenous injection of India ink and the extent of granuloma (area mm^2) in the rat, plotted against days after infection.

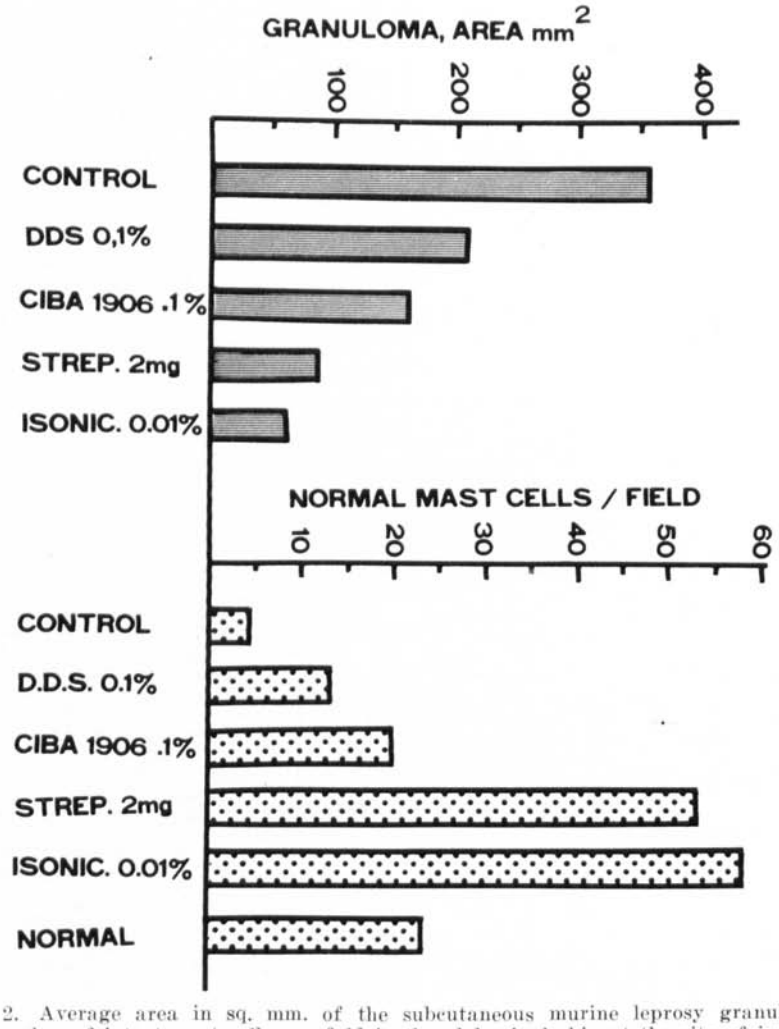

FIG. 2. Average area in sq. mm. of the subcutaneous murine leprosy granuloma and average number of intact mast cells per field in the abdominal skin at the site of infection in control and antileprosy-drug-treated rats, killed 80-83 days after infection.

(DDS), both incorporated in the diet (0.1%), showed considerable inhibiting effect on granuloma formation. At the time of autopsy only a few mast cells were counted per field in the infected controls, while the mast cell count was high in the antileprosy-drug-treated rats. The higher the activity of the drugs, the higher the mast cell count. Results are shown in Figure 2. There was no India ink accumulation in the granulomata of the infected control rats, while a slight carbon accumulation (+, ++) was registered in the lesions of animals treated with DDS and the thiourea derivative. In the small granulomata of the izoniazid- and streptomycin-treated rats, carbon accumulated as a dark spot (+++, ++++).

DISCUSSION

Histiocytes and histiocyte-like cells, imbedded in the connective

tissue ground substance and fibers, are considered as the only cellular elements contributing to the formation of the murine leprosy granuloma. The present experiments clearly demonstrate that mast cells participate widely in the physiopathology of the disease. However, while the number of histiocytes constantly increases, the number and shape of the mast cell population fluctuates characteristically during the murine leprosy granuloma formation. The number of intact mast cells reaches a peak during the pregranulomatous stage; after that they disrupt continuously at a high rate for a period of 10-20 days. This increased mast cell turnover is simultaneous with visible evolution of the granuloma. In no instance were mast cells found in the microscopic histiocyte infiltrations, but a massive accumulation was noted at the growing edges of the incipient lesions and in the area adjacent to the granuloma. In the fully developed granuloma, one seldom finds a mast cell, intact or disrupted, and in the vicinity of the lesion the number is decreased rather than normal. The question arises whether the mast cell response in murine leprosy is advantageous to the host or to the parasite, and whether these cells contribute to the defense mechanism or to the granuloma formation.

Mast cells are located predominantly in the connective tissue, around the small blood vessels, along nerves, and on serous membranes. In the rat all mast cells contain histamine, heparin and a considerable amount of serotonin ([1, 10, 11]). They participate in the production and storage of connective tissue ground substance and deposition of fibers in the connective tissue ([9, 12]). The facts observed, and deductions made from them and other facts, suggest the following hypothesis. It is known that when injury of any kind occurs to the organism, histamine and serotonin are suddenly released and these unicellular gland-like accumulations react immediately with disruption and loss of granules. The liberated biologic amines initiate the capillary response to the injury: vasodilation, increased capillary permeability, and capillary endothelial activation. There appears to be a suddenly acquired phagocytic activity of the endothelial cells. Thus, the mast cell population plays a Janus-like biologic role in the connective tissue, building ground substance and initiating a defense mechanism. Mast cell disruption is the first sign of a well-functioning defense, permitting the appearance of the two major inflammatory mediators in the damaged area, histamine and serotonin. The following activation of the defense processes ([2, 4, 13]) is largely responsible for the localization of infections and other damaging agents.

A direct relationship exists between mast cells and murine leprosy. The lesions of this disease develop exclusively in tissues rich in mast cells. The perivascular connective tissue is extremely rich in these cells and the primary lesion develops exactly at this site of the connective tissue. In our experiments, the number of intact mast cells increased considerably at a time when the lesion was limited to micro-

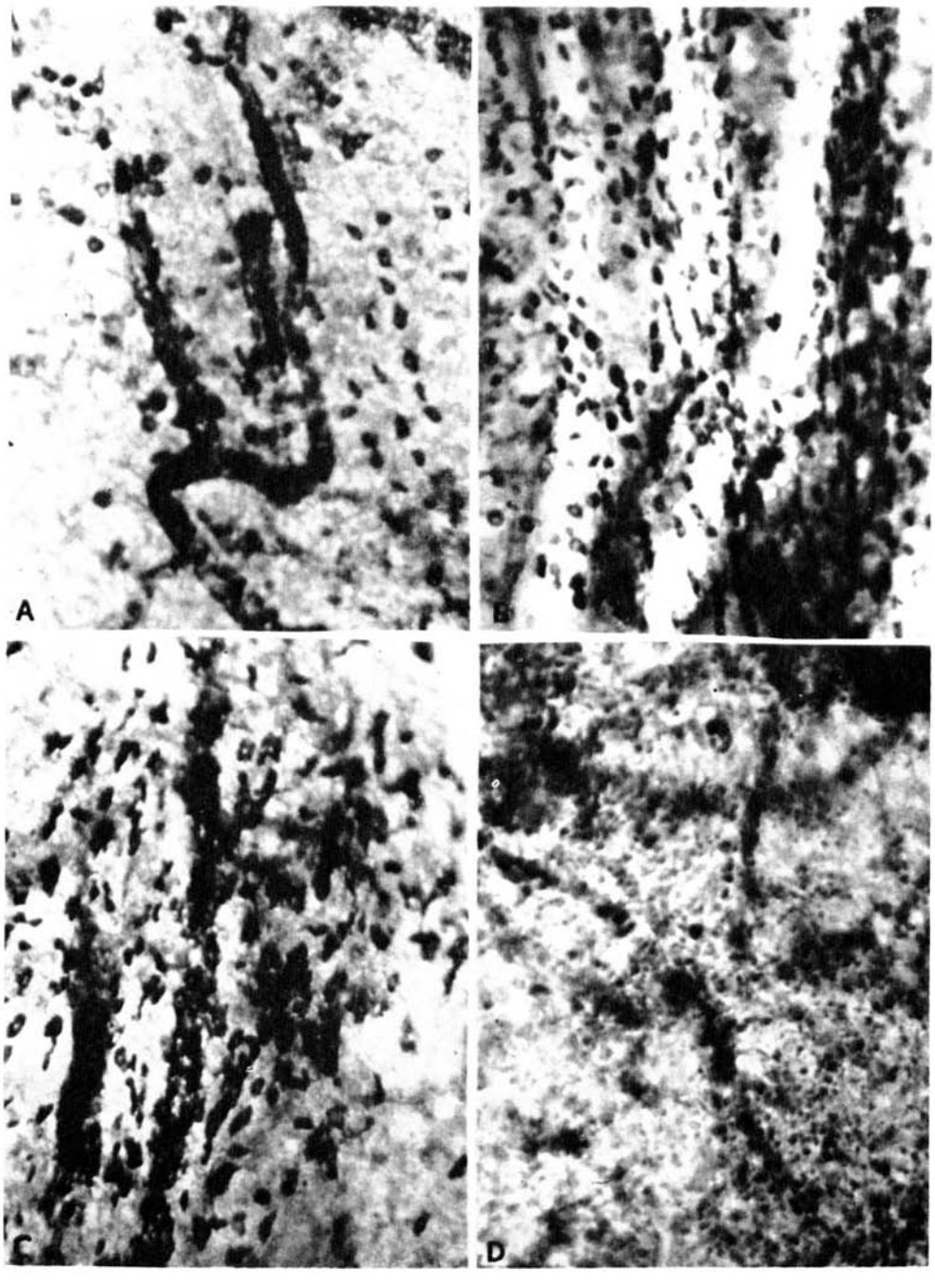

FIG. 3. **A.** Normal mast cell population in the abdominal skin of rats. **B.** Greatly increased number of mast cells in the skin 50 days after infection with murine leprosy bacilli. **C.** Giant mast cell herds in the incipient granuloma 50 days after infection. **D.** Almost complete absence of mast cells in the murine leprosy granuloma 80 days after infection. Capillaries are visualized by India ink injected intravenously. Transparent skin technics and toluidine blue-formaldehyde staining. All magnification is × 120.

scopic size. Later, when the granuloma formation was in full evolution, most of the mast cells were in a stage of disintegration, disruption and degranulation. Even at a later stage, when the granuloma was still growing, the mast cell population was far below the normal numerical level. Our investigations have shown that known chemical constituents of the mast cells, histamine, heparin, chondroitin sulfate, 5-hydroxytryptamine, and intermediate polymers of hyaluronic acid, as well as extracts from mouse mast cell tumors, are not sources of energy for *Mycobacterium lepraemurium in vitro*, but normal morphology, acid-fastness, and infectivity are considerably prolonged in the presence of heparin in saline ([8]). From results illustrated in Figure 1 no conclusion whatever can be drawn as to the relationship of mast cell proliferation to the granuloma growth. It seems that, in the pregranulomatous stage and in the early infiltration, metabolic products of bacilli, or the infection *per se* as a nonspecific stimulus, promote the multiplication and turnover of mast cells, rather than serve in an essential relationship to the promotion of granuloma growth.

It was instructive to see, by the transparent skin technics, that mast cell granules, present in great number in the connective tissue ground substance following the increased mast cell disruption, were not phagocytized by histiocytes. Maximow (1904) recognized that mast cell granules are ingested by nearby phagocytic cells. This fate of the granules was reconfirmed by several observers and recently reconsidered and discussed by Riley ([13]). But it appears not to be the case in the murine leprosy granuloma. The peculiar behavior of the phagocytic cells harboring the Stefansky bacillus in the murine leprosy lesion can be explained by the "monopolization" of histiocytes by the murine leprosy bacilli ([5]).

While it seems that mast cells do not contribute to the granuloma formation, a more acceptable explanation can be attempted regarding the role of mast cells in the defense mechanism against the invading parasite. Figure 1 shows that intravenously injected India ink accumulates intensely in the slowly progressing granuloma, exactly during the time interval when disruption of mast cells occurs in great number. Carbon accumulation at the site of injury, or tissue lesion, is a visible sign of a well-functioning defense mechanism, which includes increased capillary permeability, activation of capillary endothelium ([3]) and activation of nearby connective tissue cells ([13]). This defense process is initiated and maintained by successive waves of discharge of histamine, serotonin and heparin from the great number of continually disrupted mast cells. The vasodilator amines maintain an increased permeability, so that plasma colloids leak into the injured zone, thus permitting an afflux of immune defense factors into the infected area. Furthermore, the histamine-activated capillary endothelium acquires a strong phagocytic activity ([2, 3, 4]).

The same "activation" of the loose mesenchyma develops around

the zone of mast cell injury ([13, 14]). This stimulation of the defense mechanism permits the localization of infections, as long as it is "activated" by the mediators of mast cell origin. In earlier studies ([6]) we drew attention to the fact that a period occurs during the progress of the disease when the capillary endothelium and the phagocytic system are in a stage of constant alertness. The present results furnish experimental evidence that this endothelial and connective tissue activation is maintained during the period of increased mast cell disruption, a fact suggesting that the defense process is mediated by biologic amines of mast cell origin. At this stage of the disease no metastases occur at places remote from the site of infection. The disease is localized. However, the mast cells soon disappear completely, through an unknown mechanism, from the site of the lesion, and the capillary and connective tissue activation is no longer maintained, as visualized by the absence of carbon accumulation at the site of the lesion and in the surrounding zone. At this stage, the murine leprosy granuloma progresses rapidly, the local defense entity is no larger maintained, metastatic lesions occur, and the disease advances to its inevitable fatal outcome. This observation strongly suggests that mast cell response in murine leprosy is advantageous to the host rather than the parasite.

It was recognized in our laboratories a decade ago that chaulmoogra oil derivatives stimulate the reticuloendothelial system to increased activity ([8]). In the present experiments, drugs with established antileprosy activity not only retarded the granuloma formation considerably, but induced an altered capillary and mast cell response, in which a capillary endothelial activation was made evident by carbon accumulation in the lesions and a highly elevated mast cell count. None of the drugs inhibited granuloma formation completely, but they retarded evolution of the disease and permitted a better functioning of the natural defense processes at the site of infection. The transparent skin technics permitted a practical and quantitative evaluation of drug effects upon the evolution of murine leprosy.

Factors inducing local increase in mast cells and formation of giant mast cells, followed by massive mast cell disintegration, are still to be determined. The constant mast cell response appeared to be an integral part of the physiopathology of this chronic inflammatory process. The fate of the mast cells in the lesions is the same as that of the infected host, viz., disintegration and disappearance of the mast cells and death of the animal. In this riddle we may search for explanations of important unsolved problems in this disease.

SUMMARY

In and around the subcutaneous granulomas of murine leprosy the number of mast cells increases rapidly, reaching a peak 40 days after infection. With progression of the lesion the number of intact mast cells suddenly falls far below normal and mast cell disruption occurs

in extremely high degree. Within 50 to 80 days after transmission of the infection the number of mast cells becomes smaller and smaller. After the 80th day mast cells are seldom found in and around the granulomas. Intravenously injected India ink accumulates intensely at the site of infection at the time when mast cell disruption is maximal. This suggests that the natural defense mechanism functions well and that it is maintained by mediators of mast cell origin, viz., histamine and serotonin. It is suggested that mast cell response is advantageous to the host rather than to the parasite. When the mast cells disappear from the local lesion the disease advances to a fatal outcome. Drugs with established antileprosy effect retarded the granuloma formation, and an intense mast cell response was maintained long after these cells disappeared from infected but nontreated rats. Results were obtained by "transparent skin technics" previously developed by the authors, which permitted simultaneous visualization of capillary, mast cell and connective tissue response to the granuloma formation and the influence of drugs on the process.

RESUMEN

Después de la infección, dentro y alrededor de los granulomas subcutaneos de lepra murina, el numero de celulas cebadas aumenta rapidamente, llegando al máximo a los 40 dias después de la infección. Con el progreso de la lesión, el número de celulas cebadas intactas súbitamente cae muy por debajo de lo normal y la disrupción de celulas cebadas occurre en grados extremadamente altos. Dentró de los 50 a 80 dias después de la transmisión de la infección el número de celulas cebadas se hace menor y menor. Después de los 80 dias las celulas cebadas raramente se pueden encontrar dentro y alrededor de los granulomas. Tinta china inyectada por via endovenosa se acúmula intensamente en el sitio de la infección en el momento cuando la disrupción de las celulas cebadas es maxima. Esto sugiere que los mecanismos de defensa natural funcionan bien y que es mantenida por los mediadores de origen en las celulas cebadas, p.e.: histamina y serotonina. Se sugiere que la respuesta de celulas cebadas es ventajosa para el huesped más que para el parasito. Cuando las celulas cebadas desaparecen de la lesión local la enfermedad avanza hacia un desenlace fatal. Drogas con establecido efecto antileproso retardaron la formación del granuloma, y una respuesta intensa fué mantenida largamente después de que estas celulas desaparecieron de las ratas infectadas pero no tratadas. Los resultados fueron obtenidos por las "tecnicas de piel transparentes" previamente desarrolladas por los autores, lo que permitio la visualización simultanea de las respuestas de los capilares, las celulas cebadas y et tejido conectivo a la formación del granuloma y la influencia de las drogas en el proceso.

RESUMÉ

Dans la lèpre murine, on note une augmentation rapide du nombre des mastocytes à l'intérieur et dans le voisinage des granulômes sous-cutanés; un sommet est atteint 40 jours après l'infection.

Au fur et à mesure que la lésion progresse, le nombre des mastocytes intacts tombe rapidement sous la normale, et la destruction des mastocytes se produit à un très haut degré. En moins de 50 à 80 jours après la transmission de l'infection, le nombre de mastocytes devient de plus en plus petit. Après le 80ième jour, on trouve rarement des mastocytes à l'intérieur et autour des granulômes. L'encre de chine injectée par voie intraveineuse s'accumule en grande quantité au niveau du site de l'infection lorsque la destruction des mastocytes est la plus intense. Ce fait démontre que les mécanismes de

défense naturelle fonctionnent bien et qu'ils sont entretenus par des médiateurs qui prennent leur origine au sein des mastocytes i.e. l'histamine et la sérotonine.

Nous avançons la théorie que cette résponse des mastocytes est avantageuse pour l'hôte plutôt que pour le parasite. Lorsque les mastocytes disparaissent de la lésion locale, la maladie progresse rapidement vers une issue fatale. Des médicaments à activité anti-léprosique reconnue retarde la formulation du granulôme, et une réponse mastocytaire marquée se maintient longtemps après la disparition de ces cellules chez les rats infectés mais non traités.

Ces résultats ont été obtenus au moyen de la technique de "la peau transparente" mise au point antérieurement par les auteurs; elle permet la visualisation simultanée de la résponse des capillaires, des mastocytes ainsi que du tissu conjonctif à la formation des granulômes aussi bien que de l'influence des médicaments sur le processus.

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