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Macrophage Migration Inhibition in Experimental Diabetes (41434)

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Abstract. To study the cause of increased susceptibility to infections in diabetes, peritoneal macrophages from normal, untreated and insulin-treated diabetic rats were isolated using thioglycollate as an irritant. Alterations in the number of macrophages, their *in vitro* ability to phagocytose, and migrate from a capillary chamber in response to murine migration inhibition factor (MIF) were investigated. The macrophages from the diabetic rats exhibited decreased sensitivity to MIF and also phagocytic activity with respect to sheep red blood cells. Insulin treatment for 10 to 15 days significantly improved these characteristics toward normal.

Diabetes mellitus increases susceptibility to infections (1), which may occur as a result of inadequate disposal of foreign bacterial antigens by phagocytes like polymorphonuclear (PMN) cells and macrophages (2). These cells, particularly macrophages, are involved in the ingestion of the foreign organisms. In fact, with prior sensitization of the animal with the antigen the phagocytic activity of these cells is enhanced (3). In addition to phagocytosis the macrophages also carry out the following important roles:

(i) Identify and remove damaged cells and dead cell debris.

(ii) Act in conjunction with lymphocytes to transfer potential immunologic information of the antigen and thus initiate an immune response (4).

(iii) Recognize and control tumor cells (5, 6).

The phagocytic activity of macrophages is influenced by the initial activation of the lymphocytes, which then produce and secrete several humoral factors (7, 8). Two of these factors influence macrophages. One is a chemotactic agent which attracts the macrophages to the antigen invasion site and activates them (macrophage activation factor, or MAF (9)). The other inhibits migration of the macrophages from the antigen site (migration inhibition factor, or MIF), so that they can phagocytose and dispose of the foreign antigen "at their leisure." Macrophages reportedly contain surface receptors for MIF and produce a factor which stimulates phagocytic activity (10, 11).

The increased susceptibility to infection among diabetics, in addition to other causes may result from: (i) a decreased activation and response of leukocytes to antigens causing an inhibition in the release of the lymphokines (humoral factors produced by lymphocytes) and/or (ii) a lack of sensitivity of the phagocytes to the humoral factors (MAF and MIF). Either or both would severely compromise the immune mechanism in diabetes.

In diabetic patients migration of PMN cells to the regions of skin abrasions was considerably decreased as compared to that of nondiabetic subjects (12), indicating either a defect in the migratory ability of the phagocytes or a relatively diminished release of PMN chemotactic factor by the lymphocytes. In addition, the phagocytic activity of granulocytes obtained from diabetic patients was reported to be markedly decreased (2, 13) and showed improvement following insulin treatment. It is possible that both these effects could be attributed to functional defects in the lymphocytes as well as phagocytes in diabetes.

In this study we determined the *in vitro* sensitivity of macrophages obtained from

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diabetic animals to MIF, using a capillary tube migration assay and also studied the phagocytic activity of these cells.

Materials and Methods. Fifty young male rats (4 weeks old) of Sprague–Dawley ARS strain weighing about 70 g were obtained commercially. After one week of acclimatization, the animals were fasted overnight. Thirty-five animals were given a single dose of streptozotocin (SZ) at 65 mg/kg body weight in citrate buffer (pH 4.5) by tail vein injection. The remaining animals received citrate buffer and served as controls. Six weeks later, the animals were bled by tail vein and blood was collected in heparinized hematocrit tubes. Plasma was separated by centrifugation and glucose was determined by the glucose oxidase method, using a Beckman glucose analyzer.

The SZ-administered animals became severely diabetic as indicated by clinical symptoms of polydipsia, polyuria, and polyphagia which was accompanied by high blood glucose levels (>350 mg/dl), severe weight loss, and distended abdomens. Four to six weeks later, 14 diabetic animals were administered subcutaneously (SC) a mixture of equal parts of protamine zinc and lente insulin (Eli Lilly) diluted with saline, at a dose of 20 units per rat the first day followed by 4 units/day as a maintenance dose for 14 days before the animals were sacrificed. The remaining diabetic and control animals received daily saline (SC) injections.

Peritoneal macrophage harvestation. Peritoneal macrophages were harvested from the control, insulin-treated, and saline-treated diabetic rats, as follows. Four days prior to sacrifice the animals were injected ip with 12 ml of 3% thioglycollate (BBL, Cockeysville, Md.) in 1% glucose solution which served as a peritoneal irritant. The animals were ether anesthetized, their abdomens were shaved and thoroughly swabbed with 70% alcohol. The animals were exsanguinated via cardiac puncture. Thirty milliliters of sterile RPMI 1640 medium (Gibco) was injected into the peritoneal cavity and the abdomen was gently massaged. Using sterile instruments, a midline incision was made along the linea alba, and the peritoneal fluid was collected in sterilized beakers together with the washings of the abdominal contents. The peritoneal fluid was centrifuged at room temperature for 7 min at 200*g* and the cells were washed three times with sterile RPMI 1640 medium. The cell pellet was resuspended in RPMI 1640 medium, and the number of cells in suspension were counted in a Coulter counter.

Cellular composition of thioglycollate-elicited peritoneal exudates. Smears of peritoneal cells were stained with Wright's stain. Differential counts were made by counting a minimum of 200 cells/slide/rat.

Macrophage migration assay. The macrophage migration rates of the control, untreated, and insulin-treated diabetic rats were compared by using a modified technique described by Bloom *et al.* (14). Plain Dade microhaematocrit capillary tubes (75 × 1.2 mm, Scientific Products) were filled with cell suspensions before sealing one end with Critoseal. The capillary tubes were centrifuged, wiped with 70% alcohol, and cut slightly below the interface of packed cells and fluid. Two capillary tubes packed with cells were mounted on silicone grease in modified Sykes-Moore migration chambers. Each chamber was filled with either control or test medium and incubated at 37° for 22 hr. The basic medium was comprised of RPMI 1640 medium, supplemented with 15% dialyzed, heat-inactivated fetal calf serum (FCS) (Gibco) and antibiotics (penicillin, 100 units/ml, and streptomycin, 100 μg/ml). The test medium, in addition, contained partially purified (using an ultrafractionation technique) lyophilized migration inhibition factor (MIF) derived from cultured mouse splenic lymphocytes (15) (kindly provided by Dr. Daniel Miller, College of Pharmacy, University of Minnesota). In preliminary experiments, it was observed that this MIF actively inhibited the migration of normal rat macrophages.

Following incubation, the capillaries were projected on a screen at a magnification of 40× and the outline of the area of macrophage migration was traced. The total area of actual macrophage migration

expressed in square millimeters was determined by means of a computer at 1× (Digitizer Hewlett Packard Model 19874A). The percentage inhibition of macrophage migration in the presence of MIF was calculated by the equation as

$$\% \text{ of inhibition} = 1 - \frac{\text{average area of migration with MIF}}{\text{average area of migration without MIF}} \times 100 .$$

Macrophage culture and preparation for phagocytosis assay. Washed peritoneal exudate cells, comprising over 60% macrophages, were plated into four-chambered slides (Lab-Tek Tissue Culture Slides, Miles Laboratories, Inc., Naperville, Ill.) at 400,000 cells/chamber. Each chamber contained RPMI 1640 medium supplemented with 15% heat-inactivated FCS. After 1–2 hr, nonadherent cells were removed by two vigorous rinses with serum-free RPMI. Serum-containing medium was added to each chamber and the cells were incubated overnight. This produced a culture consisting of over 90% macrophages, as judged morphologically.

Particulate phagocytosis assays. Phagocytosis of glutaraldehyde-fixed sheep red blood cells (GSRBC) and heat-killed Baker's yeast (100° for 30 min) by cultured macrophages was assessed according to the method of Chambers and Loutit (16). Medium overlying the macrophages was removed and replaced with 0.5 ml of serum-free RPMI containing 2.5×10^7 GSRBC or yeast cells. Cells were incubated at 37° for 45 min and then washed free of yeast. GSRBC external to the macrophages were lysed by immersing the slide in 0.06 *M* NaCl for 45 sec. Slides were subsequently rinsed in phosphate-buffered saline, fixed in Bouin's, and stained with hematoxylin and eosin. Duplicate wells containing yeast or GSRBC were made. The percentage of the macrophages containing either yeast or GSRBC was determined by counting at least 200 cells/slide and the differences between groups calculated using Student's *t* test.

Results. *Cell composition of peritoneal exudates.* There was no significant difference in the total number of thioglycollate elicited peritoneal cells from diabetic or control animals (Table I).

As shown in Table II, the freshly harvested peritoneal exudates from normal untreated and insulin-treated diabetic rats contained over 60% macrophages. The proportion of other cell types, namely, neutrophils, lymphocytes, and eosinophils/mast cells, also did not show any significant difference between the control and insulin-treated and untreated diabetic rats.

Macrophage migration. In the absence of MIF in the medium, the macrophages of normal, and untreated diabetic rats migrated out of the capillary tubes (Figs. 1A and C). The macrophages of the untreated diabetic animals migrated within a mean area of 5.4 ± 0.7 mm^2. In contrast, the macrophages of insulin-treated and control rats migrated within a mean area of 6.9 ± 1.5 and 3.2 ± 0.7 mm^2, respectively. The differences between the migration of macrophages from untreated or insulin-treated diabetic rats as compared to control rats was significant ($P < 0.05$). There was no significant difference in the migration of macrophages from untreated and insulin-treated diabetic rats.

In the test medium containing MIF, the migration of macrophages from normal animals was inhibited by 71% as compared to those exposed to the basic medium not containing MIF (Figs. 1A and B). In contrast, the migration of macrophages from untreated and insulin-treated diabetic rats were inhibited 31 and 53%, respectively, by MIF (Fig. 2). The differences in inhibition between control and untreated diabetic rats and between insulin-treated and untreated diabetic rats were significant ($P < 0.001$ and <0.05, respectively). No statistical difference was found between the inhibition of migration of macrophages from control and insulin-treated diabetic rats in the presence of MIF.

The results reported above indicate that, in the presence of MIF, the migration of macrophages of untreated diabetic animals was slightly decreased, whereas those from

TABLE I. THIOGLYCOLLATE-ELICITED PERITONEAL CELLS FROM DIABETIC AND CONTROL ANIMALS

Total number of cells from								
Diabetic untreated			Diabetic insulin treated			Control		
No. of animals	No. cells $\times 10^6$	SEM $\times 10^6$	No. of animals	No. cells $\times 10^6$	SEM $\times 10^6$	No. of animals	No. cells $\times 10^6$	SEM $\times 10^6$
6	85	±11	7	93	±32	6	199	±94

insulin-treated diabetic and control animals exhibited significantly reduced migration when the lymphokine was present in the medium (Fig. 2).

Phagocytic activity of peritoneal macrophages from normal and diabetic rats. The phagocytic activity of the macrophages of control rats with respect to the untreated and insulin-treated diabetic rats is shown in Fig. 3. No significant difference was observed in the phagocytosis of yeast cells by the macrophages obtained from the three groups of rats. Using glutaraldehyde-fixed sheep red blood cells (GSRBC) the macrophages from the diabetic rats appeared to show decreased capacity to phagocytose these cells as compared to the controls ($P < 0.05$).

Discussion. The purpose of the investigation was to study the possible cause(s) of the increased incidence or susceptibility of infection in diabetes. Admittedly the rat is not an appropriate animal model in which to study infection. However, we have often observed diabetic rats with purulent discharges and hence used this animal for the present investigation.

The mononuclear phagocytes (monocyte/macrophages) are an integral part of the immune system. Alterations in their number, phagocytic capacity, and ability to respond to lymphokines (MAF and MIF) are some of the factors which may influence or affect resistance to infection.

As shown in Table I, in untreated diabetic animals the total number of thioglycollate-elicited peritoneal cells was decreased but not significantly. No explanation is evident from this observation. However, the phagocytic activity of the peritoneal macrophages from diabetic rats seemed to be somewhat lower than those of the control rats when sheep RBC were used, but not when yeast cells were added. Phagocytosis of yeast is generally considered as a model for "bacterial phagocytosis" whereas the phagocytosis of red blood cells depicts removal of "tissue debris." Our observation indicates that in diabetes the bacterial phagocytic activity of macrophages is not affected, although the clearance of effete tissue debris is somewhat compromised. It should, however, be stressed that from these *in vitro* phagocytosis assays it may be farfetched to suggest a defect in the *in vivo* phagocytic activity of macrophages in diabetic animals. In addition, the absence of homologous serum in our phagocytosis assays prevents us from commenting on the influence of opsonizing factors during *in vitro* phagocytosis or how this might correlate to *in vivo* phagocytic function.

The migratory activity of the macrophages of diabetic rats apparently is not

TABLE II. COMPOSITION OF THIOGLYCOLLATE-ELICITED PERITONEAL EXUDATE CELLS

Animal group	Macrophages (% ± SEM)	Lymphocytes (% ± SEM)	Neutrophils (% ± SEM)	Eosinophils/mast cells (% ± SEM)
Control (n = 6)	61.0 ± 2.6	15.9 ± 3.3	21.8 ± 4.8	2.3 ± 1.8
Insulin (n = 8)	68.7 ± 3.9	9.8 ± 1.7	19.0 ± 3.0	2.9 ± 1.4
Diabetic (n = 11)	64.6 ± 3.2	13.3 ± 1.7	19.8 ± 3.7	1.8 ± 0.7

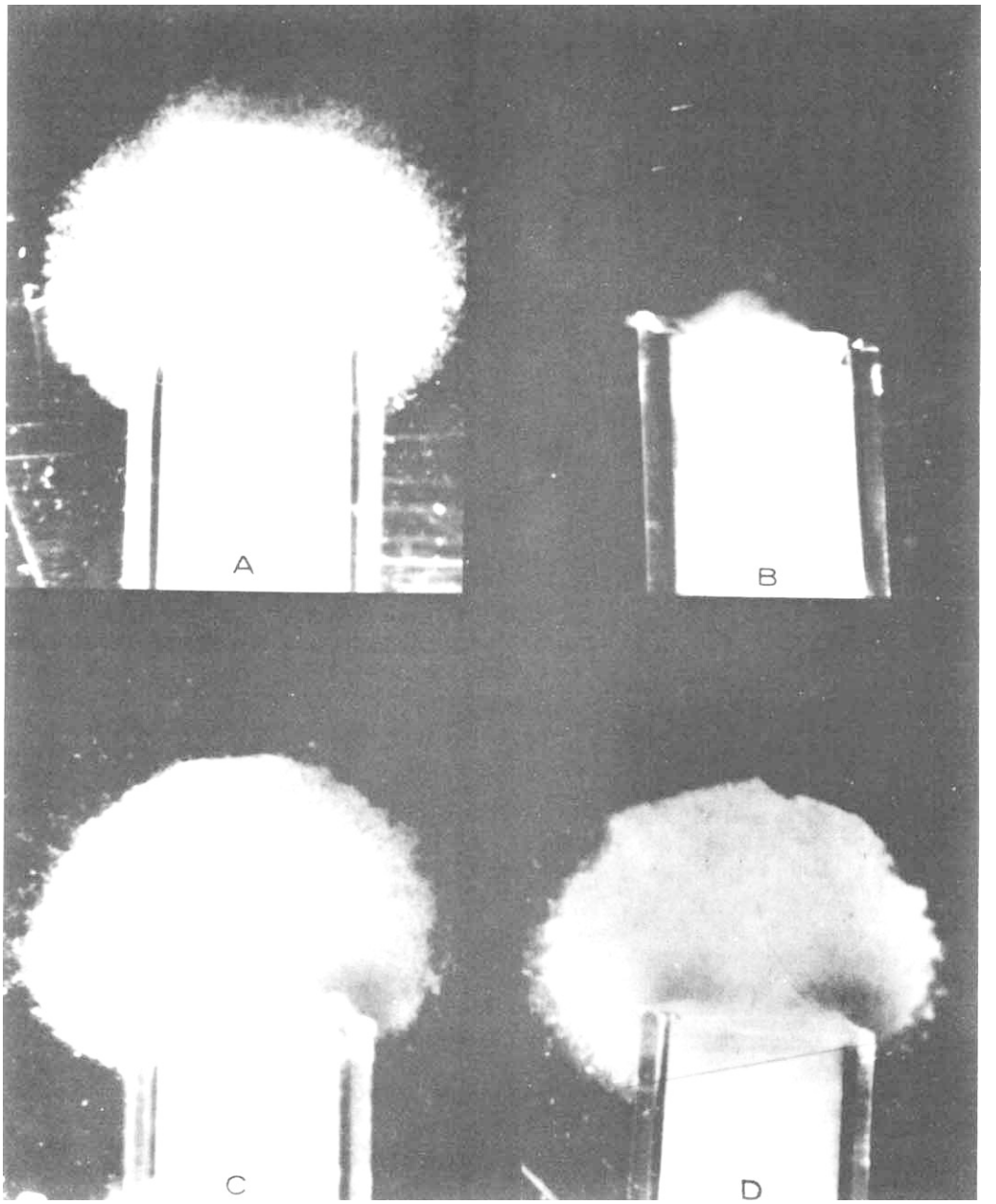

FIG. 1. Photograph showing migration out of the capillary tubing of the peritoneal macrophages obtained from normal and untreated diabetic rats in media in the absence of MIF (A and C) and in the presence of MIF (B and D).

influenced by the disease. In fact, they migrate to a greater extent than those obtained from the control animals. However, diabetes seems to affect the ability of the peritoneal macrophages to respond to MIF in contrast to those obtained from insulin-treated diabetic or control animals. Decreased sensitivity to MIF by macrophages

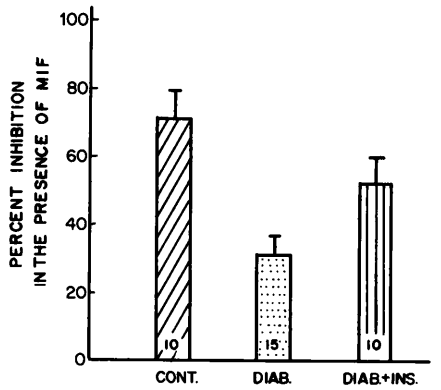

FIG. 2. Percentage inhibition in the migration of peritoneal macrophages from normal control, untreated, and insulin-treated diabetic rats in the presence of MIF in the medium. Number in the bars represent the number of rats. The line on the top of each bar denotes standard error of the mean. The *P* values between control and untreated diabetic rats and between untreated and insulin-treated rats were <0.001 and <0.05, respectively.

of diabetic animals may preclude the phagocytes from accumulating at the site of injury with resultant increase in bacterial proliferation and infection. It would be interesting to determine the dose response of insulin and/or glucose addition *in vitro* medium on the MIF responsive chemotactic action of peritoneal macrophages of diabetic and control rats. Such a study was not carried out but deserves serious consideration.

The evidence presented does not permit speculation regarding possible alteration in the production of putative lymphokines namely MIF and MAF, by the lymphocytes of the diabetic animals, but such a possibility may exist. Recent evidence indicates that lymphocytes from diabetic patients showed a lack of response to mitogens, such as phytohemagglutinin (17, 18), and concanavalin A (19).

The studies presented here indicate that, in diabetic rats, MIF has little inhibitory activity on macrophage migration. Insulin treatment for 10 to 15 days restores the sensitivity of macrophages to MIF, an action which is analogous to that of diisopropyl fluorophosphate, a specific serine esterase inhibitor (20). Further work along these lines is under way.

Improvement in macrophage sensitivity to MIF by insulin treatment is not unexpected. It is known that many morphological and functional deficiencies of the immune system in diabetes can be reversed following insulin treatment (21).

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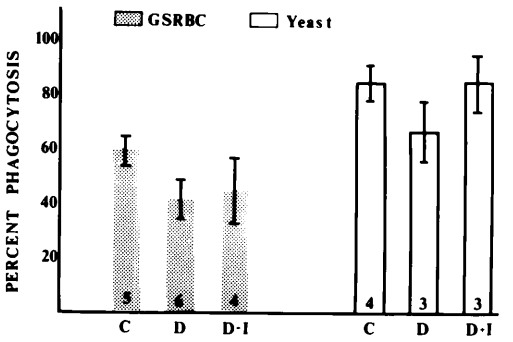

FIG. 3. Phagocytic activity of peritoneal macrophages from normal, untreated, and insulin-treated diabetic rats. Numbers within the bars represent the number of rats; the vertical lines at the top of the bar denote standard error of the mean. The difference in the phagocytic activity of GSRBC of control and diabetic rat macrophages was significant ($P < 0.05$).

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