Cytophilic Nature of Migration Inhibitory Factor Associated with Delayed Hypersensitivity* (33589)

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Macrophage migration inhibition tests show that the primary, or mediating cells of delayed hypersensitivity are sensitive small lymphocytes of peritoneal exudate (1), lymph nodes (2), peripheral blood (3). In the presence of specific antigen, they produce macromolecular substance, migration inhibitory factor (MIF), which alters the activity of macrophages. We report the effect of trypsin on the production and activity of MIF and the consequence of trypsinization of the individual cells concerned on the migration inhibition test.

Materials and Methods. Hartley colony guinea pigs were hypersensitized with complete Freund's adjuvant (Difco Lab., Detroit, Michigan) and lymphocytes separated from heparinized peripheral blood by filtration of the leukocyte suspension through packed nylon material (3). Lung alveolar macrophages of normal guinea pigs were obtained by tracheal perfusion with Hank's balanced salt solution (BSS) (4). Such cells were effective for macrophage migration inhibition tests. All cells were washed 3 times with BSS before use.

Trypsin (Difco 1:250) was dissolved in Hank's balanced salt solution (pH 7.6) in amounts of 2 and 4 mg/ml. Trypsin 2× crystallized (Mann Research Associates) was dissolved in Hank's BSS in amounts of 0.6 or 1 mg/ml. One-tenth ml of packed sensitive peripheral blood lymphocytes or normal macrophages were suspended in 2 ml of enzyme solution, incubated for 45 min at 37°, washed three times in BSS and suspended in complete medium (TCM-199 containing 20% heat-inactivated fetal calf serum and 2% L-glutamine). At the concentrations stated, both crude trypsin and crystallized trypsin produced equal effects on the cells and supernatants as listed in Tables I and II. One-third to one-half of the experiments were done with crude trypsin and the remaining with crystallized trypsin. These cells were also suspended in trypsin solution inactivated with lima bean trypsin inhibitor (TpI) (Mann Research Associates). Control cell suspensions were simultaneously incubated in BSS, washed three times in BSS and suspended in complete medium.

Cell suspensions (10^7 cells/ml) were drawn

TABLE I. Percentage Inhibition[a] by PPD[b] of Migration *in Vitro* of Untreated and Trypsinized Normal Alveolar Macrophages, and Mixtures of Such Macrophages with Untreated and Trypsinized Sensitive Peripheral Blood Lymphocytes (PBL).

Cell population	Inhibition of migration (%)
Macrophages (19)[d]	1.3 ± 0.5[c]
Trypsinized macrophages (19)	0.0 ± 0.0
Macrophages mixed with sensitive PBL (15)	25.0 ± 2.4
Macrophages mixed with trypsinized sensitive PBL (15)	29.4 ± 2.2
Macrophages mixed with sensitive PBL (20)	25.7 ± 1.7
Trypsinized macrophages mixed with sensitive PBL (20)	2.4 ± 0.9
Trypsinized macrophages mixed with trypsinized sensitive PBL (6)	0.2 ± 0.2

[a] The percentage of inhibition of migration is equal to: 100—(area of migration in experimental chamber/area of migration in control chamber) × 100.

[b] Control: migration of same cells in medium alone.

[c] Values are given as mean ± SEM.

[d] Number of animals given in parentheses.

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TABLE II. Percentage Migration Inhibition[a] of Normal Macrophages by: (a) Supernatant Fluids from Untreated and Trypsinized Sensitive Peripheral Blood Lymphocytes (PBL) Cultures, (b) Trypsinized Specific Supernatant Fluids; Migration Inhibition of Trypsinized Macrophages by Specific Supernatant Fluids.

Medium	Inhibition of migration (%)	
	Untreated macrophages	Trypsinized macrophages
Supernatant fluid from cultures of PBL with PPD[b] (6)[f]	24.9 ± 1.5[c]	
Supernatant fluid from cultures of trypsinized PBL with PPD[d] (6)	24.4 ± 2.2	
Specific supernatant fluids untreated[b] (7)	25.9 ± 2.4	
Specific supernatant fluids treated with trypsin[e] (7)	29.1 ± 2.2	
Specific supernatant fluids[b] (5)	26.5 ± 2.0	0.7 ± 0.7

[a] Calculated as in Table I.
[b] Control: supernatant fluids from cultures of PBL without PPD.
[c] Values are given as mean ± SEM.
[d] Control: supernatant fluids from cultures of trypsinized PBL without PPD.
[e] Control: supernatant fluids from cultures of PBL without PPD treated with trypsin.
[f] Number of animals given in parentheses.

into capillary tubes, which were centrifuged and the parts containing the packed cells cut and placed in Sano-Smith chambers (3). The number of packed cells was approximately 320,000. Replicate chambers were filled with 0.8 ml of complete medium containing 25 μg of tuberculin (PPD)/ml or medium alone or supernatant fluids described below. Areas of migration on the glass coverslips were determined after 24-hr incubation at 37°, by projection and planimetric measurement.

Cells studied: (a) macrophages, (b) trypsinized macrophages, (c) macrophages mixed with 20% of their number of sensitive lymphocytes or trypsinized sensitive lymphocytes, or sensitive lymphocytes incubated in trypsin inactivated by TpI, (d) trypsinized macrophages mixed with 20% of their number of sensitive lymphocytes or trypsinized sensitive lymphocytes, (e) macrophages incubated in trypsin inactivated by TpI and mixed with 20% of their number of sensitive lymphocytes.

The effect of trypsin on the production and activity of MIF in serum free media was studied. Untreated and trypsinized sensitive lymphocytes (7×10^6 cells/ml) were incubated for 20 hr at 37° in TCM-199 alone and containing 25 μg of PPD/ml. Cell-free supernatant fluids were prepared by gentle centrifugation to first settle the lymphocytes without damage. The supernatant fluid was then cleared by centrifugation at 1000*g* for 15 min. Specific supernatant fluids (containing MIF) and control supernatants prepared as above from nontrypsinized sensitive lymphocytes were incubated at 37° with 2 mg of trypsin (Difco 1:250) or 0.6 mg of crystallized trypsin/ml for 2–4 hr. At the conclusion of this incubation period these fluids still had residual trypsin activity and TpI was added in excess of that needed to inactivate trypsin present, these fluids were used as the media of capillary cultures of untreated macrophages. Specific supernatant fluids and control supernatant fluids that had not been treated with trypsin were used as the media for capillary cultures of trypsinized macrophages and also for capillary cultures of macrophages that had been previously incubated in trypsin solution inactivated with TpI.

Results. Trypsinized macrophages when suspended in medium attached readily to glass. Their area of migration was regularly greater than untreated macrophages. Table I summarizes the macrophage migration tests following trypsinization of the individual cells concerned. Trypsinization of sensitive lymphocytes did not affect their mediating ability to inhibit the migration of normal macrophages in the presence of PPD (Fig. 1 a,b). However trypsinization of macrophages

abolished the inhibitory effect on their migration by sensitive lymphocytes, in the presence of PPD (Fig. 1c,d). Macrophages incubated in trypsin inactivated with TpI responded to sensitive lymphocytes (Fig. 1 e,f). Trypsin treated macrophages when cul-

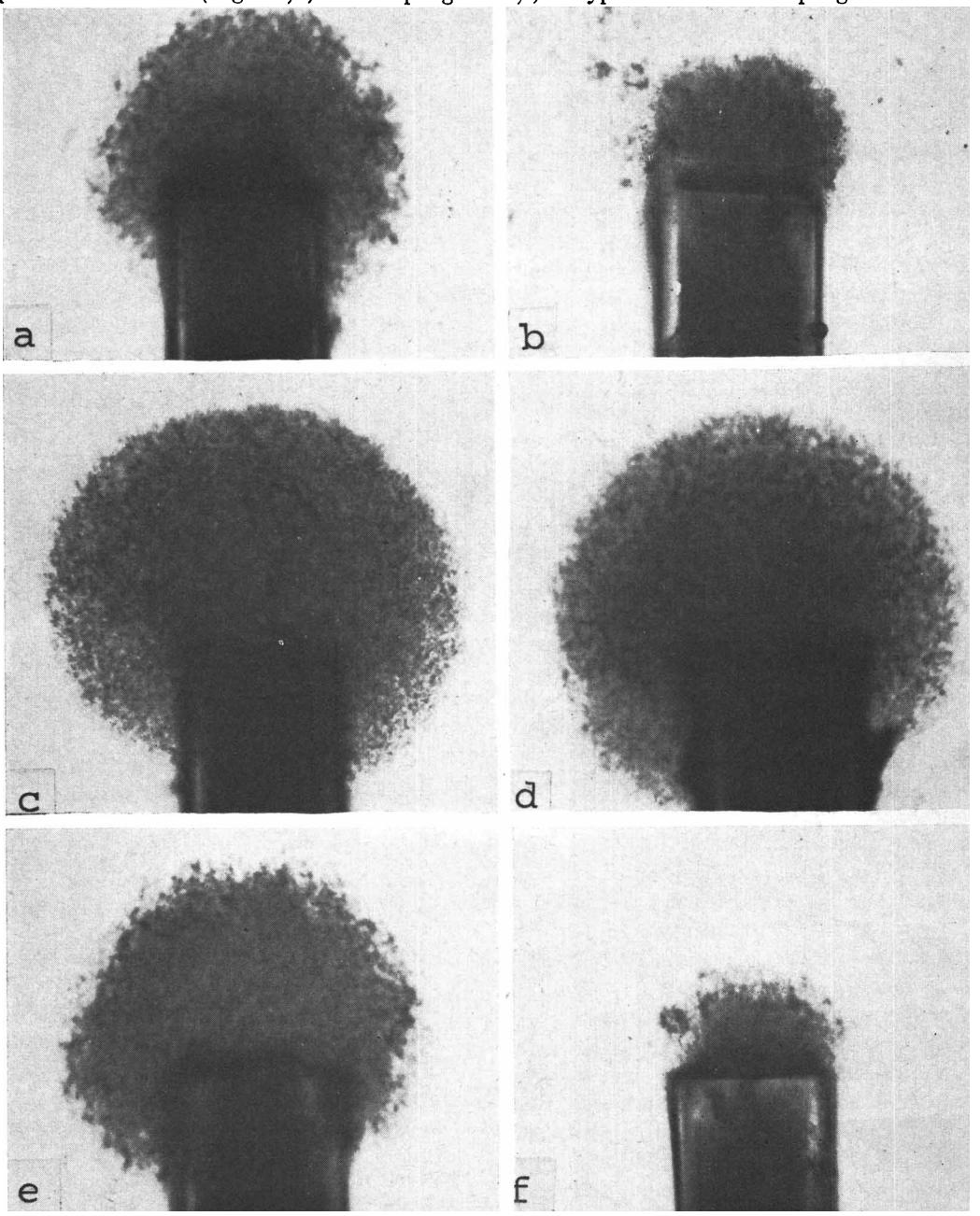

FIG. 1. Macrophage migration inhibition tests of delayed hypersensitivity: (a,b) macrophages mixed with trypsinized sensitive lymphocytes; (c,d) trypsinized macrophages mixed with sensitive lymphocytes; (e, f) macrophages incubated in trypsin inactivated by trypsin inhibitor and mixed with sensitive lymphocytes; (a,c,e) no antigen in medium; (b,d,f) specific antigen in medium; ×23.

tured for 20 hr but not 4 hr in complete medium before being placed in capillary cultures with sensitive lymphocytes regained their ability to respond.

Table II shows that trypsinized as well as untreated sensitive lymphocytes produced MIF of approximately the same inhibitory capacity. The inhibitory capacity of MIF similarly prepared in serum free media was not decreased following incubation with trypsin for 2–4 hr. The MIF did not inhibit migration of trypsinized normal macrophages but did inhibit migration of macrophages that had been incubated in trypsin inactivated with TpI. Trypsinized macrophages regained their ability to respond to MIF when they were first incubated in fresh media for 20 hr.

Discussion. David *et al* (5) reported that trypsinization of sensitive peritoneal exudate cells (mixture, of lymphocytes and macrophages) with solutions of 10 μg to 10 mg/ml of crystallized trypsin abolished their migration inhibition in the presence of specific antigen. He has since found that trypsin desensitization was reversible when such tryspinized cells were first cultured for 24 hr before being placed in capillary cultures and also stated that trypsin inactivated MIF (6). Pochyly (7) reported that trypsinized sensitive lymphocytes of lymph nodes did not inhibit the migration of peritoneal exudate cells and Bloom and Bennett (8) found that trypsinized sensitive lymphocytes of peritoneal exudate or lymph nodes did not produce MIF during the 20-hr incubation period with antigen but did when placed in medium also containing TpI.

In our experiments on the other hand, trypsin did not influence the mediating ability of sensitive peripheral blood lymphocytes or the production of MIF by such cells. We found the inhibitory capacity of supernatant fluids containing MIF produced in serum-free media, (since serum may contain trypsin inhibitors), was unchanged following incubation with trypsin for 2–4 hrs. If trypsin removed lymphocyte receptors for specific antigen or repressed production of MIF in some other way it is possible that peripheral blood lymphocytes recover from trypsin treatment much more rapidly than peritoneal or lymph node lymphocytes.

The average percentage inhibitory capacity of four supernatant fluids containing MIF decreased from 34.2 to 6.7 following absorption by untreated macrophages (50×10^6 cells/3.0 ml of specific supernatant fluid) for 1 hr at 37° while similar absorption with trypsinized macrophages (previously incubated in 0.1% crystallized trypsin) decreased the inhibitory capacity to 30.1.

Since trypsin can effect endoplasmic reticulum and ribosomal activity (9, 10), it could influence the way macrophages respond to MIF by modifying cell synthetic processes. Alternatively trypsin could act on protein containing receptor sites on macrophages for MIF. Rabinovitch (11) found that trypsin removed the receptor sites for macrophages for modified erythrocytes and suggested that macrophages synthesized such surface receptors.

Summary. Macrophage migration inhibition tests of delayed sensitivity showed that trypsinization of sensitive peripheral blood lymphocytes did not affect their mediating ability or production of migration inhibitory factor (MIF). Trypsinization of normal macrophages abolished their response to sensitive lymphocytes or MIF; their ability to respond was recovered when first cultured for 20 hr but not 4 hr. The MIF was absorbed to a greater degree by untreated than by trypsinized macrophages. These findings suggest the presence of macrophage receptors for MIF and the cytophilic nature of the latter.

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Interaction of Aggregates of Reduced Insulin with Gamma Globulin and Complement: A Pathogenetic Hypothesis* (33590)

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Berns *et al.* (1) showed that in some of the glomerular lesions found in diabetics deposits of gamma globulin and insulin are present. Burkholder (2) demonstrated that kidney slices from some diabetic patients were capable of fixing guinea pig complement *in vitro* as shown by the immunofluorescent antibody technique. Many studies of several varieties of experimental immunopathologic renal disease have shown that antigen–antibody complexes are found in glomeruli along with certain complement components (3). Involvement of the complement system seems to be required for the full expression of some of these experimental glomerular lesions. Studies of glomeruli from patients with systemic lupus erythematosus and acute glomerulonephritis (4) lend further support to the pathogenetic role of the complement system in renal damage.

These reports suggested to us the hypothesis that gamma globulin in association with insulin or insulin derivatives might be pathogenic and that insulin or some of its derivatives could produce renal and vascular lesions by a mechanism involving the complement system.

Insulin is degraded *in vivo* (5) and *in vitro* (6) by both reductive and proteolytic enzymes. This degradation has been observed in liver, kidney, adipose tissue, muscle, and the pancreas. A consistent feature of these various degradation reactions is the release of insulin A and B chains and their fragments. The biological activity of these degradation products in combination with other serum proteins has been a matter of controversy for several years (7). The data available at the present time seem to indicate that the A and B chains and their fragments have little or no insulin-like activity and no consistently demonstrable effect on the expression of insulin activity (8). However, it has been reported that the A and B chains do circulate *in vivo* (9). It also seems likely that immunoassayable insulin does not bind tightly to other serum proteins (10).

High levels of circulating, immunoassayable insulin are often associated with diabetes and/or obesity, and the combination of insulin fragments with other serum proteins substantially increases their half-life in plasma (11). However, at the present time there is no information on the structure and no quantitative data on the amounts of insulin derivatives which accumulate *in vivo*. Since diabetics frequently receive 1–2 mg of insulin daily, since the daily insulin secretion by the normal pancreas is of similar magnitude, and since insulin is continuously degraded, the ac-

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