REQUIREMENT FOR $\alpha$-L-FUCOSE ON THE MACROPHAGE MEMBRANE RECEPTOR FOR MIF*

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Macrophages play a fundamental part as effector cells in reactions of cellular immunity and hypersensitivity. Their activity in these processes is intimately connected with and may be directed by sensitized lymphocytes. Such lymphocytes when stimulated by specific antigen in vitro produce a number of soluble factors which alter the behavior of macrophages. The most studied of these substances is migration inhibitory factor (MIF), so called because of its ability to prevent the migration of macrophages in vitro from capillary tubes (1, 2). A factor indistinguishable from MIF has been shown to activate macrophages in terms of enhancing cell adherence to surfaces, ruffled membrane activity, phagocytosis, and glucose oxidation (3, 4). A different factor is chemotactic for these cells (5). The mechanism underlying the interaction of MIF with the surface of the macrophage leading to its altered behavior is unknown. Carbohydrate groups appear to be involved in this interaction as neuraminidase abolishes MIF activity (6). Further studies were carried out to investigate the role of sugars on the interaction of MIF with the macrophage by investigating the ability of a number of monosaccharides and of a glycosidase to influence the reaction. The findings that $\alpha$-L-fucose blocked the effect of MIF on macrophages and that macrophages incubated with fucosidase no longer responded to MIF taken together indicate that $\alpha$-L-fucose is an important part of the receptor for MIF on the macrophage plasma membrane.

Materials and Methods

Production of MIF.—MIF was obtained as previously described by stimulating guinea pig lymph node lymphocytes with concanavalin A (Con A, 10 $\mu$g/ml) and filtering the resulting culture fluid over a Sephadex G-100 column in 0.1 M phosphate buffer pH 7.3 containing 0.1 M NaCl (7). As a control, supernatants from lymph node lymphocytes incubated without Con A were reconstituted with the same amount of Con A and filtered over a Sephadex G-100 column. This procedure removed the Con A which binds to the Sephadex gel and provided a par-

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Abbreviations used in this paper: BSA, bovine serum albumin; Con A, concanavalin A; MEM-PS, minimum essential medium containing 85 U penicillin and 85 $\mu$g streptomycin/100 ml; MIF, migration inhibitory factor.
tially purified mediator preparation. The fractions where the MIF activity elutes containing molecules between 55,000–25,000 daltons were pooled, concentrated to 1/100 the original culture volume, and stored as aliquots at –70°C.

Assay for MIF.—Different amounts of MIF or control fraction (15 to 80 μl) were made up to 2 ml in Eagle’s minimum essential medium containing 85 U penicillin and 85 μg streptomycin/100 ml (MEM-PS) and made to contain 15% normal guinea pig serum. These media were assayed for MIF activity on normal guinea pig peritoneal exudate cells using a capillary tube migration assay (7). The assay was read at 7–12 h and at 18–24 h.

Assay for Effect of Monosaccharides on MIF Activity.—In parallel experiments, the following monosaccharides were added to the MIF and control fractions before assay at a concentration of 0.1 M: a-methyl-D-mannoside (Sigma Chemical Co., St. Louis, Mo., certified), a-D-glucose (Fisher Scientific Co., Pittsburg, Pa., certified), β-D-galactose (Fisher Scientific Co., certified), N-acetyl-β-D-glucosamine (Schwarz/Mann Div., Becton, Dickinson, and Co., Orangeburg, N. Y., certified), a-L-fucose (Schwarz/Mann, certified), and α-L-rhamnose (Eastman Organic Chemicals Div., Rochester, N. Y., certified). The sugars were chromatographically pure in two solvent systems (8). a-L-fucose had a rotation of −75.9 ± 0.2° and was found in addition to be gas chromatographically pure.

Preparation of α-L-Fucosidase from Rat Epididymis.—α-L-Fucosidase from rat epididymis was chosen because it exhibits broad specificity against a number of oligosaccharides from glycoproteins (9). The method of Carlsen and Pierce (10) with some modifications was employed using 30 g rat epididymis (stored in 20% glycerol at −20°C) for a preparation. Briefly, head sections of epididymides from 10-wk old Sprague-Dawley rats were minced in a VirTis blender (VirTis Co., Inc., Gardiner, N. Y.) in cold 0.1 M sodium acetate, 0.1 M NaCl buffer, pH 6.0 (10 ml/g tissue), and homogenized in a ground glass homogenizer. The homogenate was heated to 37°C for 1 h, the pH adjusted to pH 5.25, and the mixture heated to 60°C for 10 min and the precipitate discarded. The supernatant was made 35% of saturation in ammonium sulfate, the precipitate removed by centrifugation at 29,000 X g and the supernatant brought to 50% saturation with ammonium sulfate. After 20 h standing, the solution was centrifuged at 29,000 × g, the precipitate dissolved in, and dialyzed against 0.01 M citric acid NaOH buffer, pH 5.5 and applied to a CM-cellulose column (2 × 30 cm) equilibrated with the same buffer. The column was developed initially with 100 ml of this buffer and then a linear gradient was applied, obtained by mixing 900 ml of initial buffer and 900 ml of this buffer containing 0.25 NaCl. 10 ml samples were collected. The α-L-fucosidase activity appeared in a sharp peak. The tubes under that peak were pooled, concentrated by vacuum dialysis to 5 ml, the solution incubated for 15 min at 70°C to inactivate other glycosidases and stored in aliquots.

Assay for Glycosidases and Proteinases.—The enzyme preparation was assayed for α-L-fucosidase, at pH 6.5 in 0.05 M sodium citrate buffer, the same pH which later was used for the incubation of MIF (macrophages were incubated with the enzyme at pH 6.7 where 85% of activity at pH 6.5 is found). In addition, the activity of α-D-mannosidase, β-D-N-acetyl glucosaminidase, β-D-galactosidase, neuraminidase, and proteinase activity was determined at the same pH. This gave an indication of contaminating activities of the just mentioned enzymes. Aliquots of the enzyme solution (25–100 μl) were diluted with buffer to 0.5 ml of substrate solution. The reaction was allowed to proceed at 37°C for 30 min and stopped by addition of 3.0 ml of 0.1 M sodium glycinate pH 10.6 (10). The substrate solutions were 3 mM p-nitrophenyl-α-L-fucopyranoside for α-L-fucosidase, p-nitrophenyl-α-D-mannopyranoside (saturated solution at 4°C) for α-D-mannosidase, p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (saturated solution at 4°C) for N-acetyl-β-D-glucosaminidase, and 10 mM p-nitro-phenyl-β-D-galactopyranoside for β-D-galactosidase. The substrates were obtained from Fierce Chemical Company (Rockfort, Ill.). An enzyme unit was defined as the micromole substrate hydrolyzed per minute. It is understood that the digestion of synthetic substrates does not quantitatively reflect the action of these enzymes on natural glycoproteins. In addition, MIF fractions were tested for α-L-fucosidase activity using 25 and 12.5 μl of concentrated preparation per milliliter.
liter. N-acetyl-neuraminidase was determined by the method of Warren (11). Proteinase activity was determined by the method of Mednis and Remold (12).

**Disc Electrophoresis.**—Disc electrophoresis was performed as described by Ornstein (13) and Davis (14).

**Incubation of MIF with α-L-Fucosidase.**—Large amounts of α-L-fucosidase were used since Carlsen and Pierce were not able to hydrolyze native glycoproteins with small amounts of this enzyme (10). In our experiments, 0.75 ml of concentrated MIF and control G-100 fractions were made up to 2 ml with 0.5 M citric acid pH 6.5 containing 45 U of α-L-fucosidase/ml. The solution was incubated for 1 h at 37°C, and then put on a Sephadex G-100 column to eliminate the enzyme which eluates in the void volume. In parallel, MIF and control supernatants were incubated under the same conditions but without the enzyme and were filtered over the same columns as the enzyme treated set. Fraction III where the bovine serum albumin (BSA) marker elutes and fractions IV a and IV b (55,000–25,000 daltons) (15) of the α-L-fucosidase treated and untreated sets were pooled and concentrated to 1/100 the original volume; 25, 13, and 7 μl of these solutions were made up to 2 ml with complete tissue culture medium and tested for MIF activity.

**Incubation of Macrophages with α-L-Fucosidase.**—Oil-induced peritoneal macrophages from guinea pigs were collected in Hanks' balanced salt solution (Microbiological Associates, Bethesda, Md.) washed three times, resuspended in 0.083 M citric acid-NaOH buffer, pH 6.7, and divided into two equal parts. One part containing 0.2 ml packed cells (approximately 1.2 × 10⁸ cells) was incubated in 2 ml 0.083 M citric acid-NaOH buffer pH 6.8, the other part was incubated in the same buffer containing 32–111 U/ml α-L-fucosidase for 1 h at 37°C. The two cell pools were then washed three times with Hanks' balanced salt solution, and each divided again into two equal parts. One part was incubated with 30–50 μl of concentrated MIF containing Sephadex G-100 fractions, the other part with control fractions for 1 h at 37°C. The cells were then washed again three times with Hanks' balanced salt solution and then packed in capillary tubes and incubated in chambers containing MEM-PS in 15% guinea pig serum (without MIF or control fractions). The cells of the other part were not washed and were assayed in the presence of MIF or control fractions. In some experiments, as a control for the specificity of fucosidase, an additional set of cells was incubated with the enzyme preparation in the presence of 0.05 M α-L-fucose.

**RESULTS**

**Effects of Various Monosaccharides on MIF Activity.**—Guinea pig peritoneal macrophages were incubated with MIF in the presence of various monosaccharides. In six experiments, 0.1 M α-L-fucose, a 5-methyl pentose, consistently blocked MIF activity (see Fig. 1). In contrast, no effect was observed with other monosaccharides. A typical experiment using α-L-fucose, α-L-rhamnose (another 5-methyl pentose), and methyl-α-D-mannoside is shown in Fig. 2. Only α-L-fucose showed a significant reduction in MIF activity from 58 to 11% inhibition. Other sugars such as α-D-glucose, β-D-galactose, and N-acetyl-β-D-glucosamine did not inhibit MIF activity as seen from a typical experiment in Fig. 3.

Experiments were undertaken to investigate whether the effect of α-L-fucose was reversible. When MIF was incubated with 0.15 M α-L-fucose for 1 h and the sugar removed by extensive dialysis, the MIF was still active. Likewise, normal guinea pig peritoneal macrophages which were incubated for 1 h at 37°C with 0.1 M α-L-fucose then washed free of sugar were still inhibited by
MIF. It is evident from these experiments (see Table I) that the effect of α-L-fucose is reversible and that it had to be present throughout the assay period in order to show an effect.

Studies with α-L-Fucosidase.—Further experiments were carried out to determine whether the effect of α-L-fucose was directed to the MIF or to the macrophage. Two situations were possible: First, α-L-fucose could compete with MIF on an α-L-fucose-binding receptor on the macrophage. This would suggest that removal of α-L-fucose from MIF should result in loss of its activity. Second, glycoproteins on the macrophage surface containing α-L-fucose could function as an integral part of a MIF receptor. In this case, removal of the α-L-fucose from the macrophage surface should render the cell unresponsive to MIF. We have investigated both possibilities by determining the effect of α-L-fucosidase on either MIF or on the macrophage. It was important to determine whether the α-L-fucosidase preparation was reasonably pure since neuraminidase and proteinases can destroy MIF activity (6) and proteinases can also render macrophages temporarily unresponsive to MIF (16-19). It can be seen in Table II that there was no detectable contaminating neuraminidase or proteinase activity. There was some N-acetyl-β-D-glucosaminidase and, in one preparation, some β-D-galactosidase activity detectable. The relevance of these contaminating activities is discussed below.

The Effect of α-L-Fucosidase on MIF.—When MIF was incubated for 1 h with α-L-fucosidase, no loss of activity could be detected in three experiments. The results of a typical experiment are seen in Fig. 4 where the activity of serial
Fig. 2. The specificity of the α-L-fucose effect. A typical experiment showing the effect of 0.1 M α-L-fucose, α-L-rhamnose, and methyl-α-D-mannoside on MIF activity. MIF without sugar is designated as MIF control. The bars indicate percent inhibition of migration using 35 μl of MIF fractions per milliliter (see legend to Fig. 1). Note that only α-L-fucose prevents MIF activity.

Fig. 3. The lack of effect of other sugars on MIF activity. 20–40 μl of MIF and control fractions were used. None of these sugars inhibited MIF activity.
TABLE I
Reversibility of the \( \alpha-L \)-Fucose Effect

|                | \% Inhibition |
|----------------|---------------|
| MIF assayed on normal macrophages* | 36            |
| MIF assayed on normal macrophages in the presence of 0.1 M \( \alpha-L \)-fucose | 8             |
| MIF pretreated for 1 h with 0.15 M \( \alpha-L \)-fucose (sugar removed by dialysis) and assayed on normal macrophages | 37            |
| MIF preincubated for 1 h without sugar, dialyzed and assayed on normal macrophages | 39            |
| Macrophages which have been preincubated for 1 h in 0.1 M \( \alpha-L \)-fucose and washed three times, then assayed with MIF | 44            |
| Macrophages preincubated without sugar for 1 h and washed three times, then assayed on MIF | 35            |

* 20 \( \mu\)l Sephadex G-100 fractions concentrated to \( \frac{1}{3} \) of the original supernatant volume were added to 2 ml of culture medium. Incubations were carried out at 37°C.

TABLE II
Enzyme Activities in \( \alpha-L \)-Fucosidase Preparations after Carboxymethyl-Cellulose Chromatography at pH 6.5

| Enzyme                  | Specific activity (U/mg protein) |
|-------------------------|---------------------------------|
|                         | Preparation IV* | Preparation V |
| \( \alpha-L \)-fucosidase | 139.0             | 80.5          |
| \( \beta-D \) galactosidase | 9.1              | 0.8           |
| \( \alpha-D \) mannosidase | 0.4              | 0.8           |
| \( N' \) acetyl\( \beta-D \) glucosaminidase | 13.8          | 6.9           |
| \( N' \) acetyl\( \alpha \) neuraminidase | 0.0             | 0.0           |
| Proteinase              | 0.0               | 0.0           |

* Preparations I-III had comparable amounts of fucosidase and no detectable neuraminidase or proteinase. They were not assayed for the other enzymatic activities.

The effect of \( \alpha-L \)-fucosidase on the macrophages.—In contrast to its effect on MIF, the \( \alpha-L \)-fucosidase preparation had a marked effect on the macrophage. When macrophages were incubated for 1 h with \( \alpha-L \)-fucosidase at pH 6.7, they no longer responded to MIF. The results of four experiments are shown in Fig. 5. In these experiments, the treated and control macrophages were incubated with MIF for only 1 h and then assayed in culture medium alone for 7-12 h. When these cells were assayed with MIF for 18 h, the effect of \( \alpha-L \)-fucosidase was no longer seen (see Table III). This suggests that the monosaccharides which were removed are reconstituted on the cell surface within a few hours. It is of note that treatment with \( \alpha-L \)-fucosidase did not affect the migration of macrophages per se. The migration of \( \alpha-L \)-fucosidase treated cells
Fig. 4. Effect of α-L-fucosidase on MIF. MIF was incubated with α-L-fucosidase or buffer alone (MIF control) for 1 h and then filtered over a Sephadex G-100 column (see methods for detail). Different quantities of concentrated MIF or control fraction per milliliter were assayed. Note that α-L-fucosidase had no effect on MIF.

Fig. 5. Effect of α-L-fucosidase on macrophages. Numbers on the abscissa indicate different experiments. Cells were incubated for 1 h with α-L-fucosidase and without enzyme (control cells), washed, and incubated for a second hour in concentrated MIF or control fractions 40 μl/ml. The cells were then washed, made up in capillary tubes and percent inhibition of migration assessed at 7–12 h. The amounts of α-L-fucosidase in experiments 1–4 were 32, 111, 111, and 85 U/ml. Note that α-L-fucosidase abolished the ability of macrophages to respond to MIF in every case.
TABLE III
Recovery of Inhibitory Capacity of α-L-Fucosidase Treated Guinea Pig Macrophages within 18 h

| Treatment with α-L-Fucosidase for 1 h* | Time in MIF | % Inhibition of Migration of Macrophages |
|----------------------------------------|-------------|----------------------------------------|
|                                        | h           | Experiment I | Experiment II |
|                                        | 1           | 33           | 71           |
|                                        | + 1         | 0            | 13           |
|                                        | 18          | 48           | 92           |
|                                        | + 18        | 60           | 93           |

* 1.2 × 10^8 peritoneal exudate cells in 2 ml were incubated with and without 170 U α-L-fucosidase. The cells were washed and incubated for 1 h with MIF (40 μl in 2 ml). After 1 h one part was washed, put in capillary tubes and incubated in culture medium without MIF (i.e., time in MIF 1 h, assessment of migration inhibition at 7-12 h). The other part was assayed in capillary tubes with MIF or control fractions (i.e., time in MIF 18 h, assessment of migration inhibition after 18 h, migration was also inhibited at 7-12 h).

Further experiments indicated that the effect of the enzyme preparation was, indeed, due to α-L-fucosidase. We took advantage of the fact that this enzyme is completely inhibited by α-L-fucose (20). (We found that 66 U α-L-fucosidase/ml were completely inhibited by 0.025 M α-L-fucose.) Indeed, α-L-fucose abolished the effect of α-L-fucosidase on macrophages: macrophages were incubated in 2 ml of buffer containing 126 U α-L-fucosidase with and without 0.05 M α-L-fucose, washed, then incubated with MIF for 1 h, and assayed in capillary tubes. Whereas those that were incubated with α-L-fucosidase alone did not respond well to MIF (inhibition of migration in four experiments was 19 ± 6%), those incubated with α-L-fucosidase in the presence of α-L-fucose were still inhibited by MIF (40 ± 6%, P < 0.0005 using the paired t test). This strongly suggests that the specific action of the α-L-fucosidase on the cell surface is responsible for the abolition of the MIF response.

DISCUSSION

These studies indicate that α-L-fucose on the macrophage surface is a part of a receptor for MIF and is essential for the interaction of this factor with the macrophage. These conclusions arose from two series of experiments. The first showed that α-L-fucose blocks MIF activity reversibly whereas other sugars did not. The second demonstrated that macrophages, when incubated with α-L-fucosidase, a glycosidase which splits α-L-fucose from oligosaccharides, no longer responded to MIF.

All monosaccharides used in this study are present in the surface glycopro-
teins of the mammalian cells with exception of \( \alpha-L \)-rhamnose which is present as a glycoside in many plants. We were unable to determine whether sialic acid or \( N \)-acetyl-\( \beta-D \)-galactosamine blocked the effect of MIF since these sugars themselves occasionally inhibited migration of macrophages.

The high concentration of \( \alpha-L \)-fucose which was required to prevent the reaction suggests that the binding sites involve more monosaccharides than one \( \alpha-L \)-fucose. For example, it has been shown that the lectin Con A interacts more strongly with a dimannoside or trimannoside than with the monosaccharide methyl-\( \alpha-D \)-mannoside (21). If MIF can be shown to bind \( \alpha-L \)-fucose, affinity chromatography using this sugar might be used for the purification of this mediator.

These initial studies indicated that \( \alpha-L \)-fucose, either on MIF or on the macrophages, was necessary for the mediator-cell interaction. A glycosidase which cleaves \( \alpha-L \)-fucose from heterosaccharides was utilized to determine the location of the biologically active sugar. \( \alpha-L \)-fucosidase from rat epididymis was chosen since this enzyme is active against a variety of glycoprotein oligosaccharides (9). The finding that macrophages incubated with \( \alpha-L \)-fucosidase no longer responded to MIF indicates that the \( \alpha-L \)-fucose required for the MIF-cell interaction resides on the macrophage membrane.

\( \alpha-L \)-fucosidase is an exoglycosidase as it acts by removing the terminal non-reducing monosaccharide from the polymer (22). When several exoglycosidases are present, the substrate is sequentially digested, i.e., after each digestion, a new terminal sugar becomes available for cleavage by the appropriate enzyme. Thus, it is possible that once \( \alpha-L \)-fucose is removed from the polymer, a subterminal \( \beta-D \)-galactose could be cleaved by a contaminating \( \beta-D \)-galactosidase. Such a subterminal \( \beta-D \)-galactose might also be essential for MIF activity and is not excluded by the present studies. However, the finding that \( \alpha-L \)-fucose inhibits the effect of \( \alpha-L \)-fucosidase on macrophages indicates that at least \( \alpha-L \)-fucose is an essential terminal sugar on the macrophage receptor for MIF.

Although macrophages incubated with \( \alpha-L \)-fucosidase were unresponsive to MIF, subsequently they regained responsiveness. This suggests that the regeneration of the glycoprotein on the cell surface is rapid as has been shown by others (23–25).

There is increasing evidence for the importance of carbohydrate on the surface of cells as specific receptors for a number of biological functions. For instance, \( n \)-acetyl-neuraminic acid on the surface of some lymphocytes is important for the homing of these cells to the lymph node (26). The accumulation of lymphocytes to the node is impaired by certain viruses through the action of viral neuraminidase on the lymphocyte (27, 28). The blastogenic action of Con A on lymphocytes is prevented by specific monosaccharides which compete for the lectin binding site of the cell (29). Further, treatment of lymphocytes with neuraminidase and subsequently with galactose oxidase induces blastogenesis (30). Cell surface sugars are also important in lymphocyte cytotoxicity (31), human T cell rosette formation (32), IgE binding to mast cells (33), fertilization
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(34), and contact inhibition (35). α-L-fucose has been considered to play a role in the latter (36).

These findings are consistent with our knowledge of the plasma membrane where glycoproteins are arranged in such a way that the terminal nonreducing sugars are exposed to the environment, whereas the hydrophobic polypeptide chains are anchored in the plasma membrane (37, 38). Such molecules could pass on information from the carbohydrate receptor on the exterior to the interior of the cell.

These carbohydrates exhibit high specificity as demonstrated by the fact that the blood group A, B, H, and Lewis antigens are distinguished by only one or two monosaccharides at the chain terminal (39).

It should also be noted that α-L-fucose plays a role as an antigenic determinant in the blood group factors H, Le^A, and Le^B. These factors are characterized only by the different attachment of α-L-fucose to β-D-galactose and N-acetyl-β-D-glucosamine residues of the erythrocyte surface glycoprotein (39).

Thus, it is evident that carbohydrates on the cell surface as sites of interaction with the environment control and determine a variety of biological activities. The detection of α-L-fucose on the macrophage as a receptor for MIF is a starting point to our understanding of the interactions between lymphocyte mediators with their target cells.

SUMMARY

α-L-fucose abolishes the activity of guinea pig migration inhibitory factor (MIF) on the macrophages. Other sugars such as α-D-glucose, β-D-galactose, α-L-rhamnose, methyl-α-D-mannoside, and N-acetyl-β-D-glucosamine had no effect. The abolition of MIF activity by α-L-fucose was reversible. When macrophages were incubated with α-L-fucosidase, a glycosidase which splits terminal α-L-fucose from oligosaccharides, the macrophages no longer responded to MIF. On the other hand, MIF incubated with α-L-fucosidase was still active. These experiments strongly suggest that α-L-fucose comprises an essential part of a macrophage membrane receptor for MIF.

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