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Macrophage Migration Inhibitory Activity of Mycobacterial Growth Inhibitory Factor and the Effect of a Number of Factors on Mycobacterial Growth Inhibitory Factor Activity

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Mycobacterial growth inhibitory factor (MycoIF), which inhibits the intracellular multiplication of virulent tubercle bacilli within normal peritoneal macrophages in vitro, was tested for its ability to inhibit the migration of normal peritoneal exudate cells. The migration of peritoneal exudate cells was not inhibited by MycoIF. It was also shown that normal peritoneal macrophages infected with virulent *Mycobacterium tuberculosis*, strain H37Rv, required 72 h of incubation with spleen cell culture supernatant fluids containing MycoIF in order to inhibit intracellular bacillary multiplication. Treatment of infected macrophages with trypsin before their exposure to MycoIF abolished the ability of MycoIF to inhibit intracellular multiplication of tubercle bacilli. Incubation of infected macrophages with goat anti-mouse globulin before their exposure to MycoIF also blocked the action of MycoIF.

Mycobacterial growth inhibitory factor (MycoIF) present in the supernatant fluids from spleen cells obtained from mice immunized with viable cells of the attenuated H37Ra strain of *Mycobacterium tuberculosis*, when stimulated in vitro with viable H37Ra cells, will inhibit the intracellular multiplication of virulent H37Rv tubercle bacilli within normal mouse peritoneal macrophages (2, 3, 8, 14). In the preceding reports (2, 3) we have described some of the characteristics of MycoIF. Results based on studies using puromycin and enzymes (3) have suggested that MycoIF is protein in nature and that terminal sialic acid residues may be present. Filtration of MycoIF-rich supernatant fluids on Sephadex G-150 columns demonstrated that MycoIF eluted in a molecular weight range of 20,000 to 35,000 (3).

With the demonstration of MycoIF's inhibitory activity on the intracellular multiplication of virulent tubercle bacilli and the determination of some of its physical and chemical characteristics, we thought it of interest to further explore whether MycoIF had macrophage migration inhibitory (MIF) activity.

We also have examined the interaction of MycoIF with peritoneal macrophages in an attempt to gain some insight into how macrophages exposed to MycoIF develop an enhanced capacity to inhibit the intracellular multiplication of tubercle bacilli.

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MATERIALS AND METHODS

The procedures for the production of MycoIF by spleen cells obtained from mice immunized with the attenuated H37Ra strain of *M. tuberculosis*, as well as the procedure for maintaining normal mouse peritoneal macrophages infected with virulent tubercle bacilli in vitro, have been previously described in detail (2, 3).

The macrophage migration inhibition technique was an adaptation of that used by David et al. (4) as modified for use with mouse cells by Neiburger and Youmans (12).

The mouse migration inhibition technique (12) utilizes NCTC 135 tissue culture medium. To validly control the MIF assays in our studies, the control supernatant fluids containing MIF were produced in NCTC 135 + 15% horse serum instead of in RPMI 1640 + 5% human serum (8). MycoIF-containing supernatant fluids and fractions produced in RPMI 1640 + 5% human serum were dialyzed against NCTC 135, supplemented with horse serum, and sterilized by filtration before their testing for MIF activity.

The migration of macrophages in supernatant fluid from non-antigen-stimulated lymphocytes was considered 100% migration. The MIF activity in the supernatant fluid was determined by comparison with control: percentage of inhibition = 100 − [(area of migration with antigen × 100)/(area of migration without antigen)]. The statistical significance of the difference between test and control migration was determined by Student's t test.

Determination of minimum exposure time of infected macrophages to MycoIF. MycoIF was added to infected macrophages on the day of infection. Twenty-four hours later the medium was removed by aspiration from a set of cultures and re-

placed with fresh macrophage maintenance medium alone. This procedure was repeated at 48 and 72 h after infection, using sets of infected macrophages.

Trypsin treatment of infected macrophages. Infected cover-slip macrophage cultures were incubated for 30 min in RPMI 1640 + 1 mg of trypsin per ml (Worthington Biochemical Corp.) or RPMI 1640 + 5 mg of trypsin per ml in a 5% CO_2-37 C incubator. The macrophage cultures were then rinsed four times in 37 C RPMI 1640 and transferred to tissue culture bottles containing MycoIF.

Treatment of trypsin-treated macrophages with a macrophage heat eluant. Peritoneal cells from 30 nonimmunized mice were collected as previously described (2). After centrifugation, the pellet, consisting of 1.2×10^8 cells, was resuspended in 1.5 ml of Hanks balanced salt solution, incubated at 56 C for 30 min, and centrifuged, and the supernatant fluid was collected (macrophage heat eluant).

Infected macrophages on cover slips were incubated with 5 mg of trypsin per ml as described above. The macrophage heat eluant was subsequently overlaid onto each infected trypsinized and washed cover slip macrophage culture and incubated for an additional 30 min in a 5% CO_2-37 C incubator. The cover-slip macrophage cultures were then rinsed once in 37 C RMPI 1640 and transferred to bottles containing MycoIF plus medium. An additional control was included in the experiment to exclude the possibility that trypsinized macrophages were contaminated with residual trypsin after rinsing and that any loss in inhibitory activity was not due to a direct effect of the trypsin on MycoIF. As an added control, supernatant fluid containing MycoIF was incubated with three trypsinized cover-slip macrophage cultures for 1 h. These fluids were then transferred to a fresh tissue culture bottle, and untreated infected macrophage cultures were introduced.

Treatment of infected macrophages with antisera. Infected normal peritoneal macrophages were incubated with the immunoglobulin G fraction of goat anti-mouse globulins (lot 7441, Cappel Laboratories, Downington, Pa.) diluted 1:4 or 1:32 in RPMI 1640 for 30 min. The infected cover-slip macrophage cultures were then rinsed four times in 37 C RPMI 1640 and transferred to bottles that held supernatant fluids containing MycoIF supplemented with medium.

RESULTS

Inhibition of migration of normal peritoneal exudates by MycoIF and Sephadex G-150 gel filtration fractions of MycoIF. Experiments were performed to determine whether MycoIF or the gel filtration fractions described in the preceding paper (3) that had produced significant intracellular inhibition of tubercle bacilli in vitro would inhibit the migration of normal peritoneal exudates. Significant inhibition of migration when compared with controls has been correlated by others with the presence of MIF (1, 5).

The percentage of migration inhibition of the cells caused by the various supernatant fluids when compared with the migration produced by control supernatant fluids is given in Table 1. It can be concluded from these results that supernatant fluids containing MycoIF did not inhibit the migration of peritoneal exudates. Further more, none of the MycoIF-containing gel filtration fractions showed inhibitory activity.

Effect of time of exposure of infected macrophages to MycoIF on the inhibition of intracellular multiplication of virulent tubercle bacilli. Infected macrophages incubated in the presence of MycoIF only for the first 24 or 48 h of their infection did not inhibit the intracellular multiplication of tubercle bacilli (Table 2). Significant inhibition of the intracellular multiplication of tubercle bacilli was only observed when infected macrophages were incubated with MycoIF for 72 h.

Trypsinization of macrophages before exposure to MycoIF. Experiments were performed to determin whether the inhibition of growth produced by MycoIF within infected macrophages could be blocked by trypsinization of the infected macrophages before exposure to MycoIF. Incubation of infected macrophages with 5 mg of trypsin per ml completely eliminated the ability of MycoIF to produce statistically significant intracellular inhibition within these macrophages (Fig. 1).

Treatment of trypsin-treated macrophages with a macrophage heat eluant. Experiments were also conducted to attempt to restore the ability of trypsinized infected macrophages to inhibit intracellular growth by incubating them, before their exposure to MycoIF, in a heat eluant from nonimmunized macrophages incubated at 56 C. No restoration of ability to inhibit the intracellular multiplication of virulent tubercle bacilli was noted.

Trypsinization of macrophages after exposure to MycoIF. Experiments were also done in which the MycoIF was removed from a set of untreated macrophages cultures 72 h after infection. The macrophages were then treated with 5 mg of trypsin per ml, rinsed in 37 C RPMI 1640, and then returned to fresh medium without additional MycoIF for the remainder of the experiment. Treatment of infected macrophages with trypsin after 72 h of exposure to MycoIF resulted in some loss in the ability of the macrophages to inhibit the intracellular multiplication of tubercle bacilli (Fig. 2).

Effect of goat anti-mouse total globulin antisera on the activity of ability of MycoIF. Treatment of infected macrophages with 1:4

TABLE 1. *Inhibition of migration of normal peritoneal exudates by mouse spleen cell supernatant fluids and gel filtration fractions*

Mouse spleen cell supernatant fluids and gel filtration fraction[a]	Increase (%)[b]	Lymphocyte culture medium[c]	Percent migration inhibition when compared with the migration produced by:[d]				
			N-O	N-Ra	Ra-O	N-Ra fraction III	N-Ra fraction IV
N-O		NCTC 135					
N-Ra		NCTC 135	0				
Ra-O		NCTC 135	0				
Ra-Ra		NCTC 135		43 $P < 0.025$[e]	50 $P < 0.005$		
Unfractionated N-Ra control	26	RPMI 1640		21 $P < 0.05$			
N-Ra column fraction III	24	RPMI 1640		24 $P < 0.05$			
N-Ra column fraction IV	30.6	RPMI 1640		20 $P < 0.05$			
Unfractionated Ra-Ra control (expt 1)	62.3	RPMI 1640		0	0		
Ra-Ra column 2, fraction I (expt 1)	29.4	RPMI 1640				0	
Ra-Ra column 2, fraction II (expt 1)	57.9	RPMI 1640					0
Unfractionated Ra-Ra control (expt 2)	66.2	RPMI 1640		0	0		
Ra-Ra column 2 fraction I (expt 2)	37.8	RPMI 1640				0	
Ra-Ra column fraction II (expt 2)	68.6	RPMI 1640					0
Ra-Ra column 2, fraction III (expt 2)	37.2	RPMI 1640					0

[a] N-O, Supernatant fluids from nonimmunized mouse spleen cells cultured in 15% horse serum and 85% NCTC 135 in the absence of antigen. N-Ra, Supernatant fluids from nonimmunized mouse spleen cells cultured in 15% horse serum and 85% NCTC 135 in the absence of viable H37Ra cells. Ra-O, Supernatant fluids from H37Ra-immunized mouse spleen cells cultured in 15% horse serum and 85% NCTC 135 in the absence of antigen. Ra-Ra, Supernatant fluids cultured as above in the presence of viable H37Ra cells. Unfractionated N-Ra control, An aliquot of supernatant fluids produced in RPMI 1640 prepared for gel filtration, obtained from the sample just before application to the column. N-Ra column, fractions III and IV, Pooled fractions from $(NH_4)_2SO_4$-treated N-Ra supernatant fluids chromatographed by gel filtration on Sephadex G-150. Unfractionated Ra-Ra control (expt 1), An aliquot of supernatant fluids produced in RPMI 1640 prepared for gel filtration, obtained from sample just before application to the column. Ra-Ra column 2, fractions I and II (expt 1), Pooled fractions from 55% $(NH_4)_2SO_4$-treated Ra-Ra supernatant fluids chromatographed twice by gel (reference 3, Table 4) filtration on Sephadex G-150. Results of experiment 1 are similar to those of experiment 2, and fractions from the two experiments are also similar (reference 3, Table 4). Unfractionated Ra-Ra control (expt 2), An aliquot of supernatant fluids prepared for gel filtration, obtained from sample just before application to the column (reference 3, Table 4). Ra-Ra column 2, fractions I and II, and III (expt 2), Pooled fraction from 55% $(NH_4)_2SO_4$-treated Ra-Ra supernatant fluids chromatographed twice by gel filtration on Sephadex G-150 (reference 3, Table 4, Fig. 4).

[b] Percentage of increase in the number of macrophages containing 10 or fewer tubercle bacilli when compared with the same value obtained from O-O cultures (reference 3, Table 4).

[c] Spleen cells were cultured at a density of 1.5×10^7 cells/ml in 15% horse serum and 85% NCTC 135 tissue culture medium or 5% human serum and 95% RPMI 1640. All supernatant fluids were dialyzed against NCTC 135 and adjusted to 15% serum before introduction to Mackaness-type chambers.

[d] Percentage of migration inhibition of normal peritoneal exudate cells.

[e] Statistical significance determined by Student's *t* test.

TABLE 2. *Inhibitory response of normal mouse macrophages infected with virulent tubercle bacilli when exposed for various periods of time to MycoIF*

Source of mouse spleen cells[a]	In vitro exposure to viable H37Ra cells (mg)	No. of hours of supernatant fluid contact with infected macrophages[b]	No. of macrophages with ≤10 bacilli[c]	No. of macrophages with >10 bacilli[d]	Increase (%)[e]	Statistical significance of differences in the no. of intracellular tubercle bacilli when compared with:		
						O-O[f]	Ra-O[g]	Ra-Ra[h]
O[f]	0	0	168	132				
H37Ra immunnized	0	72	156	144	−7.2	0.966[i] $P < 0.5$ > 0.25[j]		
H37Ra immunized	0.12	24	171	129	1.7	0.061 $P < 0.5$ > 0.25	1.512 $P < 0.25$ > 0.1	37.95 $P < 0.005$
H37Ra immunized	0.12	48	164	136	−2.4	0.107 $P < 0.75$ > 0.5	0.428 $P < 0.75$ > 0.5	45.04 $P < 0.005$
H37Ra immunized	0.12	72	241	59	43.4	40.92 $P < 0.005$	53.79 $P < 0.005$	

[a] Spleen cells were cultured at a density of 1.5×10^7 cells/ml. Supernatant fluids from 1.5×10^8 spleen cells were added on the day of infection.

[b] Spleen cell supernatant fluids were added to infected macrophage cultures on the day of infection and incubated for the period of time shown.

[c] Sum of the three macrophage cover slip "under 10" values of one parameter.

[d] Sum of the three macrophage cover slip "over 10" values of one parameter.

[e] Percentage of increase in the number of infected macrophages containing 10 or fewer tubercle bacilli when compared with the same value obtained from O-O cultures.

[f] O-O, Infected macrophages cultured in the absence of spleen cell supernatant fluids.

[g] Ra-O, Supernatant fluids from H37Ra-immunized mouse spleen cells cultured in the absence of antigen.

[h] Ra-Ra, Supernatant fluids from H37Ra-immunized mouse spleen cells cultured in the presence of viable H37Ra cells and then incubated for 72 h with infected macrophages.

[i] Chi-square value.

[j] Probability value; the chi-square value is significant if $P \leq 0.05$ and very significant if $P \leq 0.005$.

dilution of goat anti-mouse globulin before exposure to MycoIF completely inhibited the ability of MycoIF to inhibit intracellular multiplication of tubercle bacilli (Table 3).

DISCUSSION

The results add further support to the previous observations of Klun and Youmans (8) that MycoIF and MIF are different substances. These results also support the thesis that delayed hypersensitivity and cellular immunity in tuberculosis are independent phenomena (19). The results of other investigators (12, 15, 16, 19) provide additional evidence that does not support a relationship between delayed hypersensitivity and cellular immunity to infection with *M. tuberculosis*. In addition, Simon and Sheagren (18) have reported that supernatant fluid from antigenically stimulated bovine gamma globulin-immunized lymphocytes exhibited substantial MIF activity without enhancing the ability of macrophages to kill *Listeria monocytogenes*. Osebold et al. (13) also have recently examined the relationship between cellular immunity and delayed hypersensitivity in listeriosis. Their results suggest that antimicrobial cellular immunity to infection can occur as a phenomenon independent of delayed hypersensitivity.

The experiments in which infected macrophages were exposed to MycoIF for various periods of time after infection showed that only macrophages incubated with MycoIF for 72 h inhibited intracellular multiplication of virulent tubercle bacilli. Removal of macrophages from MycoIF at 48 h resulted in no observable intracellular inhibition. The final 24 h of the 72-h incubation period, therefore, appears to be

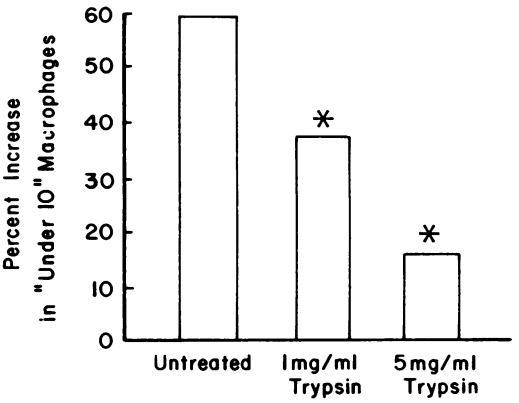

FIG. 1. *MycoIF activity after treatment of macrophages with trypsin. Asterisk indicates statistically significant difference from controls.*

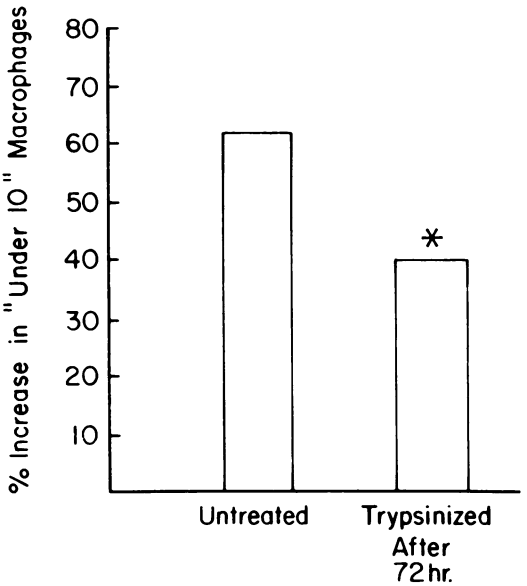

FIG. 2. *Effect of trypsinization of macrophages after exposure to MycoIF on MycoIF activity. Asterisk indicates statistically significant difference from controls.*

the most important in providing the macrophages with the ability to inhibit intracellular multiplication of tubercle bacilli.

Nathan et al. (11) showed that correlates of macrophage activation (cell adherence and glucose-1 oxidation) only rose to elevated levels after 72 h of macrophage contact with MIF-rich fractions. However, if the MIF-containing medium was then removed and replaced with fresh medium, the elevated macrophage levels began to drop back to control levels after 24 h. Within 48 h after removal of the activating factor, the macrophages returned to control levels. If the MycoIF-induced intracellular inhibition observed in our infected macrophages was strictly dependent upon "macrophage activation," intracellular macrophage enzyme levels should have returned to normal levels within 96 to 120 h after infection, and yet inhibition of mycobacterial growth continued for another 4 to 5 days.

The data involving trypsinization of the macrophage before exposure to MycoIF indicate that there are trypsin-sensitive membrane components or "receptors" that are important in the macrophage interaction with MycoIF. Their loss would result in the inability of the macrophage to interact with MycoIF. It is conceivable that the changes caused on the macrophage surface by interaction with MycoIF, whether it is a binding of MycoIF or a conformational change in receptors caused by MycoIF, may be important in maintaining the macrophage's ability to inhibit intracellular growth. Whether this surface "alteration" is stable or results in further changes in the macrophage is not known. However, trypsinization of infected macrophages after 72 h of incubation with MycoIF resulted in the loss of ability to inhibit intracellular growth. It is possible that trypsin treatment of MycoIF-exposed macrophages may have directly inactivated MycoIF attached to the cell membrane.

The inability of the MycoIF to induce intracellular inhibition of growth of tubercle bacilli in macrophages treated with goat anti-mouse sera implies that the antisera in some way blocked the interaction between MycoIF and the macrophage surface. However, it is not possible, from the results of this experiment, to determine whether the blocking was specific or nonspecific. If this was a specific inhibition, it might indicate that the receptor involved for inducing the macrophage by MycoIF was a cytophilic antibody. Nonspecific blocking by the antisera may have resulted from binding to immunoglobulins on the membrane which are in close proximity to MycoIF receptors. This may have resulted in steric hindrance and an inability of MycoIF to bind with its own receptors.

Heise et al. (7) suggested that cytophilic antibody may play an important role in macrophage migration inhibition because they could resensitize trypsinized macrophages with heat eluant derived from sensitive macrophages. Our attempts to restore the trypsinized macrophages' ability to interact with MycoIF by incubating them with macrophage heat eluants were not successful.

David et al. (6) have reported that pretreat-

TABLE 3. *Activity of MycoIF on goat anti-mouse gamma globulin-treated mouse peritoneal macrophages infected with virulent tubercle bacilli*

Source of mouse spleen cells[a]	In vitro exposure to viable H37Ra cells (mg)	Infected macrophage treatment	No. of macrophages with ≤10 bacilli[b]	No. of macrophages with >10 bacilli[c]	Increase (%)[d]	Statistical significance of differences in the no. of intracellular tubercle bacilli when compared with		
						O-O[e]	Ra-O[f]	Ra-Ra[g]
O[e]	0	Untreated	172	128				
O	0	1:4 goat[h] anti-mouse gamma globulin antisera	173	127	0.05	0.0096[i] $P > 0.9$[j]		83.1 $P > 0.005$
H37Ra immunized	0	Untreated	142	158	−17.5	6.01 $P < 0.025$ > 0.01		
H37Ra immunized	0.12	Untreated	271	29	57.5	84.55 $P < 0.005$	129.2 $P < 0.005$	
H37Ra immunized	0.12	1:4 goat anti-mouse gamma globulin antisera	135	165	−21.6	9.13 $P < 0.005$		140.8 $P < 0.005$
H37Ra immunized	0.12	1:32 goat[h] anti-mouse gamma globulin antisera	170	130	−1.2	0.027 $P < 0.9$ > 0.75		87.28 $P < 0.005$

[a] Spleen cells were cultured at a density of 1.5×10^7 cells/ml. Supernatant fluids from 1.5×10^8 spleen cells were added on the day of infection.

[b] Sum of the three macrophage cover slip "under 10" values of one parameter.

[c] Sum of the three macrophage cover slip "over 10" values of one parameter.

[d] Percentage of increase in the number of macrophages with 10 or fewer tubercle bacilli when compared with the same value obtained from O-O cultures.

[e] O-O, Infected macrophages cultured in the absence of spleen cell supernatant fluids.

[f] Ra-O, Supernatant fluids from H37Ra-immunized mouse spleen cells cultured in the absence of antigen.

[g] Ra-Ra, Supernatant fluids from H37Ra-immunized mouse spleen cells cultured in the presence of viable H37Ra cells and incubated with untreated infected macrophages.

[h] Infected macrophages incubated for 30 min with goat anti-mouse gamma globulin antisera (immunoglobulin fraction) diluted 1:4 or 1:32 with RPMI 1640 before exposure to supernatant fluids.

[i] Chi-square value.

[j] Probability value; the Chi-square is significant if $P \leq 0.05$ and very significant if $P \leq 0.005$.

ment of sensitive peritoneal exudate cells with trypsin abolished the ability of specific antigens to inhibit their migration. Remold (17) reported that treatment of macrophages with α-L-fucosidase also abolished their responsiveness to MIF. Our data as well as the results of these investigators and others (9) would suggest that the macrophage membrane surface contains various "receptors," which may be involved in interactions with lymphokines.

It should be stated that our present evidence for a relationship between MycoIF and the in vivo cell-mediated immunity to tuberculosis is strictly dependent upon in vitro observations. Until MycoIF can be used to protect mice against in vivo challenge with virulent H37Rv tubercle bacilli, no direct correlations can be made between our in vitro observations of cell-

mediated intracellular inhibition and the in vivo phenomena surrounding cell-mediated immunity to tuberculosis.

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