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Conditions for Production, and Some Characteristics, of Mycobacterial Growth Inhibitory Factor Produced by Spleen Cells from Mice Immunized with Viable Cells of the Attenuated H37Ra Strain of *Mycobacterium tuberculosis*

DAVID L. CAHALL[1] AND GUY P. YOUMANS*

Department of Microbiology, Northwestern University Medical School, Chicago, Illinois 60611

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Mycobacterial growth inhibitory factor (MycoIF), found in supernatant fluids of mouse spleen cell cultures that have been stimulated in vitro with homologous antigen, inhibited the intracellular multiplication of virulent tubercle bacilli within normal mouse peritoneal macrophages in vitro. Antigenically stimulated H37Ra-immunized mouse spleen cells required 72 h of incubation to produce supernatant fluids that would cause intracellular inhibition. Supernatant fluids from 48-h mouse spleen cell cultures were not able to produce intracellular inhibition. Investigation of the culture conditions showed that at least 1.0% human serum was required in the tissue culture medium for the production of MycoIF by spleen cells from immunized mice. MycoIF activity was noted only in supernatant fluids from spleen cell cultures incubated with antigen for 72 h. MycoIF was nondialyzable and unaffected by freezing, lyophilization, or incubation at 60 C for 30 min. However, MycoIF was inactivated after incubation at 80 C for 30 min. MycoIF was unaffected by low hydrogen ion concentrations (pH 7 to 12), but exposure to higher hydrogen ion concentrations (pH 6, pH 5) significantly decreased MycoIF activity, and exposure to pH 4 to 2 abolished all activity. Supernatant fluids diluted 1:32 were still able to produce significant intracellular inhibition of growth of virulent tubercle bacilli.

Tissue culture techniques have permitted investigators to examine the relationship between the lymphocyte and the macrophage in cell-mediated immunity to infection with facultative intracellular parasites. Patterson and Youmans (11) demonstrated that the addition of spleen cells from mice immunized with viable cells of the attenuated H37Ra strain of *Mycobacterium tuberculosis* to normal peritoneal macrophages infected with virulent *M. tuberculosis* cells resulted in the inhibition of intracellular mycobacterial multiplication. They also reported that the cell-free supernatant fluids from cultures of immune spleen cells that had been incubated with mycobacterial cells caused identical intracellular mycobacterial growth inhibition when added to infected macrophages. This activity was attributed to a mycobacterial growth inhibitory factor (MycoIF).

Klun and Youmans (9), using a similar system, noted that viable H37Ra cells, mycobacterial ribonucleic acid (RNA), or purified protein derivative (PPD) was capable of inducing lymphocytes from mice immunized with viable attenuated mycobacterial cells to produce mycobacterial growth inhibitory supernatant fluids. However, only H37Ra cells and mycobacterial RNA were capable of inducing mycobacterial RNA-immunized lymphocytes to produce supernatant fluids with inhibitory activity. When spleen cells from mice immunized with mycobacterial RNA were stimulated with PPD, supernatant fluids were produced that had only the low inhibitory activity noted with supernatant fluids from spleen cells obtained from nonimmunized animals after stimulation with PPD.

Klun et al. (8) also have reported the apparent lack of identity between migration inhibitory factor (MIF) activity and MycoIF. They found that lymphocyte supernatant fluids from H37Ra-immunized mice did not consistently contain both MIF and MycoIF. The absence of MIF was noted in some supernatant fluids that inhibited intracellular growth of tubercle bacilli in macrophages, and MycoIF activity was not present in some supernatant fluids that inhibited the migration of normal macrophages. These results suggested that MIF and

[1] Present address: Department of Microbiology and Immunology, Temple University Medical School, Philadelphia, Pa. 19140.

MycoIF were different substances and that tuberculin hypersensitivity and immunity to tuberculosis were mediated by different lymphokines.

Apart from the above information on the ability of MycoIF to inhibit the intracellular growth of virulent tubercle bacilli within normal mouse peritoneal macrophages, very little is known about the nature of MycoIF. Studies were undertaken, therefore, to define some of the chemical and physical characteristics of MycoIF and to determine some of the culture conditions required by lymphocytes for its production.

MATERIALS AND METHODS

Mice. Male C57Bl/6 mice (Jackson Laboratories, Bar Harbor, Me.) weighing approximately 18 to 22 g were housed 10 per cage in air-conditioned quarters and fed food and water ad libitum.

Mycobacterial preparations. *M. tuberculosis* strains H37Ra and H37Rv were maintained by weekly subculture of pellicles on a modified Proskauer and Beck synthetic medium (14). The attenuated strain, H37Ra, was used for immunization of mice and for antigenic stimulation of lymphocyte cultures. The virulent strain, H37Rv, was used to infect normal mouse peritoneal macrophages in vitro.

Vaccination. Mice were immunized subcutaneously with 1 mg (10^6 to 10^7 viable particles) (moist weight) of viable H37Ra cells in 0.2 ml of 0.01 M phosphate buffer, pH 7.0. H37Ra-immunized mice were used as sources of spleen cells 28 to 38 days after vaccination.

Collection and culture of macrophages. Peritoneal macrophages from nonimmunized mice were collected and cultured on sterile cover slips according to the methods of Chang (3) and Patterson and Youmans (12). The only modification was the substitution of RPMI 1640 for NCTC 135 in the macrophage maintenance medium (MMM).

Preparation of spleen cell cultures. Mouse spleens were collected aseptically and prepared for culture according to the method of Klun and Youmans (9). The spleen cells were adjusted to 1.5×10^7 cells/ml in a lymphocyte culture medium (LCM), which was composed of 95% RPMI 1640, 5% fresh, heat-inactivated human serum (skin test negative to PPD), 50 U of penicillin per ml, and 2 μg of Fungizone per ml. The cell suspension was pipetted into tissue culture tubes (Falcon Plastics, No. 3033) (3 ml/tube), after which 0.12 mg of viable H37Ra cells in a 0.1-ml volume was added to each culture tube. The spleen cell cultures were incubated at a 5° angle for 72 h in a 37 C, 5% CO_2 incubator. After incubation, the lymphocyte cultures were centrifuged at 4 C at $200 \times g$ for 10 min, and the supernatant fluid was decanted aseptically and pooled. Supernatant fluids were transferred to tissue culture bottles (4-oz [about 120-ml] borosilicate bottles; Foster-Forbes Glass Co., Marion, Ind.) for assay for MycoIF activity or were stored by freezing at −70 C.

Infection of macrophages. A suspension of *M. tuberculosis* strain H37Rv was prepared as previously described (14). To insure that all large clumps of bacilli were removed, the suspension was filtered through a double thickness of sterile 9-cm Celeritas filter paper. After filtration, the turbidity was adjusted to 8 to 10 Klett units with filter no. 54 (540 nm) in a Klett-Summerson photoelectric colorimeter. Subsequently, 2.5 ml of bacilliary suspension was added to 100 ml of medium consisting of 75% RPMI 1640 and 25% horse serum (GIBCO, Grand Island, N.Y.). Ten milliliters of this suspension was added to each petri dish containing six to eight cover-slip macrophage cultures. The macrophage cultures were placed in a humid 5% CO_2, 37 C incubator, and phagocytosis of the mycobacteria was allowed to occur. After 1 h the infecting medium was aspirated, and the cover slips were washed four times with physiological saline warmed to 37 C. Three macrophage culture cover slips were then transferred to a sterile tissue culture bottle containing 10 ml of supernatant fluid from 1.5×10^8 cultured spleen cells and additional medium to provide a final concentration of 55% RPMI 1640, 40% horse serum, 5% beef embryo extract (GIBCO), plus penicillin and Fungizone. The macrophage cultures were incubated in a humid 5% CO_2, 37 C incubator.

Maintenance and termination of macrophage experiments. At 3 and 6 days after infection, the culture medium was removed and replaced immediately with fresh medium (MMM) without additional lymphocyte supernatant fluids. Nine days after infection three cover slips were removed from each tissue culture bottle, rinsed in physiological saline, and fixed in neutral buffered formalin for 15 min. The cover slips were stained in steaming carbol fuchsin and counter-stained with hematoxylin. Cover slips were mounted in Permount (Fisher Scientific Co., Fair Lawn, N.J.) for microscopic examination.

Determination of inhibition of intracellular mycobacterial growth within macrophages. One hundred infected macrophages on each of the three cover slips were examined. The macrophages microscopically were divided into two groups: those containing 10 or fewer tubercle bacilli ("under 10") and those containing more than 10 tubercle bacilli ("over 10"). These numerical values were chosen after microscopic examination of infected macrophages. Infection with tubercle bacilli results in a macrophage infection rate of 18 to 25% with an average of one to three bacilli per macrophage. Results have shown that tubercle bacilli within macrophages in the presence of supernatant fluids containing MycoIF may replicate once or twice; however, 85 to 95% of the macrophages never contain more than eight to ten bacilli. Exponential multiplication of tubercle bacilli is readily observed in macrophages incubated in the presence of supernatant fluids which do not contain MycoIF. The number of tubercle bacilli within these macrophages will vary between 20 and too numerous to count.

To provide an indicator of the mycobacterial growth inhibitory effect of supernatant fluids, the total "under 10" value for infected macrophages incu-

bated in the absence of supernatant fluids was used as a base line. When the total "under 10" value of a test was compared with the same values obtained from the control, a percentage of increase in the number of macrophages with 10 or fewer tubercle bacilli could be obtained. The percentage of increase in the number of macrophages containing 10 or fewer tubercle bacilli was calculated by the following formula:

$$\text{Percentage of increase} = \left(\frac{\text{"under 10" value of test}}{\text{"under 10" value of control}} - 1.0 \right) \times 100$$

This percentage of "under 10" macrophages is, of course, directly related to the degree of inhibition of the intracellular growth of the virulent mycobacteria.

Statistical analysis was performed by the method of chi square.

RESULTS

Demonstration of the activity of MycoIF on the intracellular multiplication of virulent tubercle bacilli within normal mouse peritoneal macrophages. Initial experiments were carried out to confirm the production of MycoIF by mouse spleen cells.

The MycoIF in supernatant fluids from immunized mouse spleen cells cultured in the presence of viable H37Ra cells markedly increased (63.2%) the number of macrophages containing 10 or fewer tubercle bacilli (Table 1). This difference in intracellular inhibition was highly statistically significant ($P < 0.005$). This table also shows the effect of supernatant fluids from cultures of spleen cells obtained from nonimmunized mice.

Effect of length of incubation time of lymphocyte cultures on production of MycoIF. After 24 h of incubation in complete LCM, specifically stimulated spleen cell cultures were centrifuged at 200 × *g* for 10 min at 27 C and the supernatant fluids were collected. The same procedure was repeated with another group of spleen cell cultures after 48 h of incubation in complete LCM. After a total of 72 h of incubation at 37 C, supernatant fluids also were collected and tested for the presence of MycoIF. Only the supernatant fluids from spleen cell cultures incubated for 72 h inhibited the intra-

TABLE 1. *Activity of MycoIF on the intracellular multiplication of virulent tubercle bacilli within normal mouse peritoneal macrophages in vitro*

Source of mouse spleen cells[a]	In vitro exposure to viable H37Ra cells (mg)	No. of macrophages with ≤10 bacilli[b]	No. of macrophages with >10 bacilli[c]	Increase (%)[d]	Statistical significance of differences in no. of intracellular tubercle bacilli when compared with:			
					O-O[e]	N-O[f]	N-Ra[g]	Ra-O[h]
O[e]	0	177	123					
Nonimmunized mice	0	191	109	7.9	1.73[i] $P < 0.25$ > 0.5[j]			
Nonimmunized mice	0.12	209	91	18	7.43 $P < 0.01$ > 0.005	2.43 $P < 0.25$ > 0.1		
H37Ra-immunized mice	0	187	113	5.6	0.698 $P < 0.5$ > 0.25			
H37Ra-immunized mice	0.12	289	11	63.2	120.53 $P < 0.005$	100.04 $P < 0.005$	75.59 $P < 0.005$	105.76 $P < 0.005$

[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 three macrophage cover slip "under 10" values of one parameter.

[c] Sum of 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] N-O, Supernatant fluids from nonimmunized mouse spleen cells cultured in the absence of antigen.

[g] N-Ra, Supernatant fluids from nonimmunized mouse spleen cells cultured in the presence of viable H37Ra cells.

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

[i] Chi-square value.

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

cellular multiplication of tubercle bacilli (Table 2).

Effect of serum in lymphocyte tissue culture medium on the production of MycoIF. Further experiments were carried out to determine the effect of serum in the LCM on the production of MycoIF. Spleen cell cultures were prepared in LCM containing 5, 3, or 1% human serum and also in LCM containing 1% human serum plus additional glutamine (2 mM/ml) and LCM composed only of RPMI 1640 or RPMI 1640 plus additional glutamine (2 mM/ml). Only H37Ra spleen cells cultured in LCM containing human serum inhibited the intracellular multiplication of tubercle bacilli. The production of MycoIF in LCM containing 1% human serum was significantly less than when 3 or 5% serum was used (Table 3).

Effect of exposure to various temperatures on the inhibitory activity of MycoIF. Aliquots of active supernatant fluids were exposed to various temperatures for 60 min and immediately cooled in an ice bath. The supernatant fluids were then tested for MycoIF activity. The intracellular inhibition produced by supernatant fluids was unaffected by exposure to 4, 27, or 60 C for 60 min (P values ranged from >0.1 to >0.75) (Fig. 1). However, incubation at 80 C for 60 min abrogated the inhibitory activity of MycoIF ($P < 0.005$).

Effect of dialysis on the inhibitory activity of MycoIF. Active lymphocyte supernatant fluids were dialyzed against 600 volumes of double-distilled water, pH 7.5, for 36 h at 4 C and then against 50 volumes of RPMI 1640 for 12 h. The supernatant fluids were then sterilized by filtration through 0.45-μm membrane filters (Millipore Corp.). The ability of MycoIF to inhibit the intracellular multiplication of tubercle bacilli was completely unaffected by dialysis.

Effect of freezing or lyophilization on the inhibitory activity of MycoIF. Experiments were performed to determine whether supernatant fluids containing MycoIF could be produced in large quantities and stored for long periods of time without affecting its ability to produce intracellular inhibition. Aliquots of active supernatant fluids were frozen at −70 C or lyophilized in vacuo. Lyophilized samples were stored at 4 C in sealed desiccant jars. Seven days after treatment, frozen supernatant fluids

TABLE 2. *Inhibitory activity of MycoIF in supernatant fluids from H37Ra-immunized mouse spleen cells cultured for various periods of time*

Source of mouse spleen cells[a]	In vitro exposure to viable H37Ra cells (mg)	Duration of in vitro spleen cell culture incubation[b] (h)	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		163	137				
H37Ra-immunized mice	0	72	168	132	3	0.16[i] $P < 0.75$ > 0.5[j]		
H37Ra-immunized mice	0.12	24	175	125	7.3	0.975 $P < 0.5$ > 0.25	0.33 $P < 0.75$ > 0.5	84.63 $P < 0.005$
H37Ra-immunized mice	0.12	48	183	117	12.2	2.73 $P < 0.1$ > 0.05	1.54 $P < 0.25$ > 0.1	74.01 $P < 0.005$
H37Ra-immunized mice	0.12	72	273	27	67.4	101.53 $P < 0.005$	94.33 $P < 0.005$	

[a] Spleen cells were cultured and supernatant fluids were added as in Table 1.
[b] Spleen cells were cultured in lymphocyte culture medium 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] Expressed as in Table 1.
[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 for 72 h.
[i] Chi-square value.
[j] Probability value; the chi-square value is significant if $P \leq 0.05$ and very significant if $P \leq 0.005$.

TABLE 3. *Inhibitory activity of MycoIF in supernatant fluids produced by H37Ra-immunized mouse spleen cells cultured in vitro in various lymphocyte culture media*

Source of mouse spleen cells[a]	In vitro exposure to viable H37Ra cells (mg)	Lymphocyte culture medium[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] (5% HS)	Ra-O[h] (glu)	Ra-O[i] (RPMI)	Ra-Ra[j] (5% HS)
O[f]	0		104	196						
H37Ra immunized	0	5% HS + 95% RPMI 1640	126	174	21.1	3.41[k] $P < 0.1 > 0.05$[l]				
H37Ra immunized	0.12	5% HS + 95% RPMI 1640	287	13	175	245.88 $P < 0.005$	201.37 $P < 0.005$			
H37Ra immunized	0.12	3% HS + 97% RPMI 1640	283	17	172	233.2 $P < 0.005$	189.31 $P < 0.005$			0.56 $P < 0.5 > 0.25$
H37Ra immunized	0.12	1% HS + 99% RPMI 1640	269	31	158	209.4 $P < 0.005$	151.5 $P < 0.005$			7.94 $P < 0.005$
H37Ra immunized	0.12	1% HS + 99% RPMI 1640 + 2 mM glutamine/ml	262	38	151	174.89 $P < 0.005$	161.9 $P < 0.005$			13.39 $P < 0.005$
H37Ra immunized	0	100% RPMI 1640	59	241	−43.3	17.05 $P < 0.005$	35.08 $P < 0.005$			
H37Ra immunized	0.12	100% RPMI 1640	74	226	−28.9	7.18 $P < 0.01 > 0.005$	20.2 $P < 0.005$		2.17 $P < 0.25 > 0.1$	391.5 $P < 0.005$
H37Ra immunized	0	100% RPMI 1640 + 2 mM glutamine/ml	58	242	−44.3	17.89 $P < 0.005$	36.24 $P < 0.005$			
H37Ra immunized	0.12	100% RPMI 1640 + 2 mM glutamine/ml	151	149	45	15.0 $P < 0.005$	4.19 $P < 0.05 > 0.025$	63.5 $P < 0.005$		156.04 $P < 0.005$

[a] Spleen cells were cultured and supernatant fluids were added as in Table 1.
[b] Spleen cells were cultured for 72 h in the various lymphocyte culture media listed. HS, Human serum.
[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] Expressed as in Table 1.
[f] O-O, Infected macrophages cultured in MMM without spleen cell supernatant fluids.
[g] Ra-O, Supernatant fluids from H37Ra-immunized mouse spleen cells cultured in 5% human serum + 95% RPMI 1640 in the absence of antigen.
[h] Ra-O (glu), Supernatant fluids from H37Ra-immunized mouse spleen cells cultured in 100% RPMI 1640 + 2 mM glutamine per ml in the absence of antigen.
[i] Ra-O (RPMI), Supernatant fluids from H37Ra-immunized mouse spleen cells cultured in 100% RPMI 1640 in the absence of antigen.
[j] Ra-Ra (5% HS), Supernatant fluids from H37Ra-immunized mouse spleen cells cultured in 5% human serum + 95% RPMI 1640 in the presence of viable H37Ra cells.
[k] Chi-square value.
[l] Probability value, the chi-square value is significant if $P \leq 0.05$ and very significant if $P \leq 0.005$.

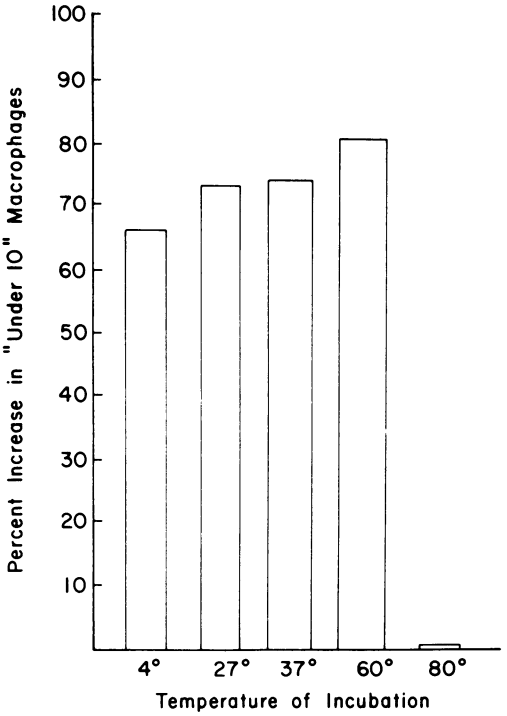

FIG. 1. *Effect of exposure to various temperatures on the activity of MycoIF.*

were thawed and lyophilized supernatant fluids were reconstituted in double-distilled water to original volume and sterilized by filtration. The inhibitory activity of MycoIF was not reduced by freezing or by lyophilization. It is also very interesting to note that the inhibitory activity of MycoIF was not affected by storage at −70 C for 1 year.

Effect of exposure to various hydrogen ion concentrations on the inhibitory activity of MycoIF. Aliquots of active supernatant fluids were adjusted to various hydrogen ion concentrations with 1 N HCl and 1 N NaOH and incubated in a 37 C water bath for 60 min. The supernatant fluids were then readjusted to pH 7.2 and sterilized by filtration. Exposure to low hydrogen ion concentration (pH 8 to 12) did not statistically enhance or decrease the inhibitory activity of MycoIF when compared with control supernatant fluids (pH 7.0) (Fig. 2). However, exposure of active supernatant fluids to pH 6.0 or 5.0 caused a statistically significant reduction in the inhibitory activity of MycoIF ($P < 0.005$). Supernatant fluids incubated at pH 4.0, 3.0, or 2.0 actually enhanced the intracellular growth of tubercle bacilli within the macrophages (Fig. 2).

Effect of dilution on the inhibitory activity

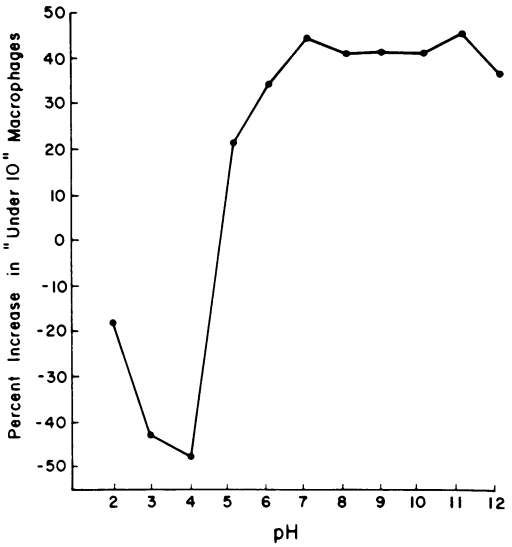

FIG. 2. *Effect of exposure to various hydrogen ion concentrations on the activity of MycoIF.*

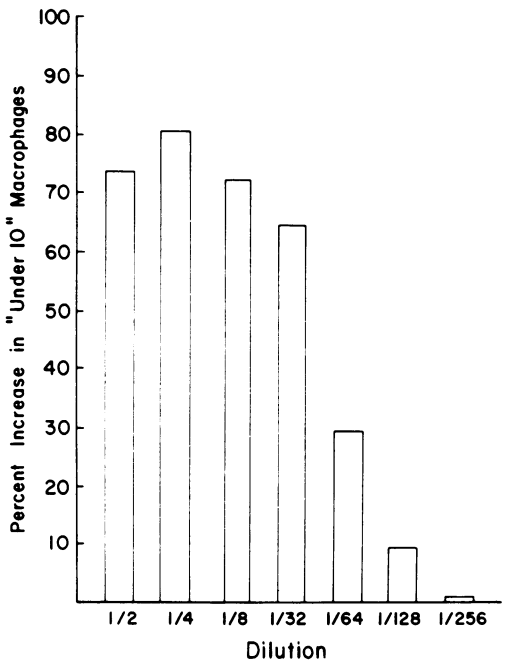

FIG. 3. *Effect of dilution on the activity of MycoIF.*

of MycoIF. Active supernatant fluids were serially diluted with RPMI 1640 + 5% human serum (1:2 to 1:128). Ten milliliters of the undiluted and of each diluted supernatant fluid was separately added to tissue culture bottles containing 8 ml of supplemental medium, resulting in approximately a further twofold dilution.

Infected macrophage cultures were introduced into each bottle. The dilution figures shown in Fig. 3 are the approximate values for the final dilution of supernatant fluids when they were added to the tissue culture bottles. No statistically significant decrease in growth inhibitory activity was noted until a dilution of 1:64 was reached.

DISCUSSION

Initial experiments in this investigation confirmed that H37Ra-immunized mouse spleen cells stimulated in vitro with viable H37Ra produced MycoIF as previously reported (9, 11).

The in vitro extracellular multiplication commented upon by various investigators (5, 13) has not been a problem in our system because of the inability of tubercle bacilli to multiply in the MMM. The horse serum present in MMM contains sufficient transferrin of high iron-binding capacity which chelates available free iron. The unavailability of sufficient iron will result in the inhibition of extracellular myobacterial growth (6, 7, 9).

This study also has demonstrated that only supernatant fluids from antigenically stimulated H37Ra-immunized spleen cells, which had been cultured for 72 h, were capable of producing MycoIF. Since there was no observable MycoIF activity in supernatant fluids from spleen cell cultures maintained for only 48 h, this would indicate that MycoIF synthesis only occurs, or that demonstrable levels of MycoIF only appear, during the last 24 h of antigenic stimulation. Adler et al. (1) have reported that phytohemagglutinin-stimulated mouse spleen cells show a sudden increase in blast transformation between 24 and 48 h, indicating that macromolecular activity was significantly enhanced only after the first 24 h of antigenic stimulation. Although the production of MycoIF was not correlated with blast transformation in our investigation, antigenic stimulation of H37Ra-immunized spleen cells did cause a significant increase in [^{3}H]thymidine incorporation (unpublished data). Therefore, it is possible that the presence or production of MycoIF may be dependent upon blast transformation and the concomitant enhanced macromolecular activity of sensitized spleen cells.

Various attempts to produce MycoIF in the absence of serum were unsuccessful. A minimum of 1% fresh human serum in the LCM was required for observable MycoIF activity. Gazit and Harris (4) reported that they were able to maintain the viability of CBA and BALB/c mouse spleen cells in mixed leukocyte cultures composed of RPMI 1640 supplemented with 2 mM glutamine per ml. Supernatant fluids produced under these conditions in our study did not inhibit intracellular multiplication of tubercle bacilli. Other investigators (1, 10) have reported the prolonged survival of mouse spleen cells cultured in RPMI 1640 supplemented with fresh human serum. This, together with our results, indicates that mouse spleen cells require an enriched tissue culture medium supplemented with fresh human serum to remain viable for extended periods of time.

The physical characterization experiments indicated that MycoIF was nondialyzable and unaffected by freezing, lyophilization, or incubation at 60 C for 30 min. However, MycoIF was inactivated after incubation at 80 C for 30 min. MycoIF was unaffected by low hydrogen iron concentrations (pH 7 to 12), but exposure to higher hydrogen iron concentrations (pH 6, pH 5) significantly decreased MycoIF activity or abrogated its ability to produce intracellular inhibition (pH 4 to 2). Other experiments demonstrated that when supernatant fluids were diluted 1:32 they were still able to produce significant intracellular inhibition of tubercle bacilli.

This information on the physical characteristics and conditions for production of MycoIF have enabled us to pursue studies to further characterize MycoIF. These results will be presented in the following paper (2).

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