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Partial Characterization of a Factor Extracted from Sensitized Lymphocytes That Inhibits the Growth of *Mycobacterium tuberculosis* Within Macrophages In Vitro

R. TURCOTTE,* Y. DES ORMEAUX, AND A. G. BORDUAS

Centre de Recherche en Immunologie, Institut Armand-Frappier, C.P. 100, Laval-des-Rapides, Ville de Laval, Québec, Canada H7N 4Z3

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Spleen lymphocytes of BCG-immunized mice contain a soluble factor that inhibits in vitro the growth of the H37Rv strain of *Mycobacterium tuberculosis* within normal peritoneal macrophages. The water-soluble extracts of sensitized lymphocytes, disrupted by freezing and thawing, although less active than the corresponding viable cells retained a significant growth-inhibiting activity. Dialysis against distilled water, lyophilization, exposure to ribonuclease and deoxyribonuclease, and storage at −20°C of the water-soluble extracts did not affect their antimycobacterial activity, whereas extracts heated at 100°C were completely devoid of such an activity. All the inhibiting activity was recovered in the void volume of the column after chromatography on Sephadex G-200. Water-soluble constitutents of sensitized lymphocytes did not affect BCG grown in vitro, and on repeated treatments of tuberculous mice they led to a negligible protection against pulmonary tuberculosis. Preliminary observations seem to indicate that other soluble factors in lymphocytes of BCG-sensitized mice have the capacity to potentiate in vitro the phagocytic activity of normal macrophages.

It is now well established that sensitized lymphocytes control infection by facultative or obligate intracellular parasites within normal macrophages maintained in tissue culture. Indeed, Patterson and Youmans (17) demonstrated that lymphocytes from mice immunized with the H37Ra strain of *Mycobacterium tuberculosis* when added to normal macrophages infected in vitro with the H37Rv strain inhibited the intracellular growth of this microorganism. Likewise, several investigators observed similar inhibiting effects when cell-free supernatants obtained by exposing in vitro sensitized lymphocytes to the specific sensitizing antigen were used on monolayers of macrophages infected not only with the homologous but also with unrelated intracellular pathogens (9, 11, 13, 21). Therefore, it would appear that sensitized lymphocytes, upon stimulation with the specific antigen, release a soluble factor that can inhibit the intramacrophage growth of various intracellular microorganisms.

The exact nature and the mechanism of action of this bacterial growth inhibitory factor are still unknown. Some investigators (7, 14, 16) have postulated that it is a lymphocyte mediator that activates normal macrophages, thus enhancing their microbicidal capacity. On the other hand, it has not yet been possible with the technique currently available to distinguish the macrophage activating factor from the macrophage migration inhibitory factor (16), whereas other workers presented some evidence suggesting that the bacterial growth inhibitory factor and the macrophage migration inhibitory factor were different substances (3, 10, 22). The physicochemical characteristics of a mycobacterial growth inhibitory factor (MycoIF) present in cell-free supernatants of antigenically stimulated lymphocytes have been reported recently by Cahall and Youmans (1, 2).

The present paper deals with the characterization of a MycoIF that, instead of being liberated in vitro by exposing sensitized lymphocytes to the specific antigen, was extracted directly from spleen lymphocytes of mice immunized with BCG (Bacillus Calmette-Guérin). This factor was chromatographed on Sephadex G-200, and its stability towards different enzymes, dialysis, lyophilization, and temperature variation, as well as its activity on pulmonary tuberculous infection in mice and on in vitro cultures of BCG, were examined.

MATERIALS AND METHODS

Mycobacterial strains. The Montreal substrain of BCG was used as the immunizing agent. The virulent H37Rv strain of *M. tuberculosis* was used for the in vitro infection of macrophages. Both strains were cultured as a surface pellicle on Sauton medium and were 14 days old when harvested. A suspension of BCG containing 10 mg (moist weight) of bacilli per ml of Sauton medium (1:4) was prepared and kept frozen at −50°C until used. With H37Rv, the technique of Sansonnens (20) was used to eliminate bacterial clumps, and thus well-dispersed bacilli were obtained for the infection of macrophages. Suspensions of dispersed H37Rv containing 10 mg (moist) weight) of bacilli per ml of Sauton medium (1:4) were prepared, distributed in vials, and kept frozen at −50°C. The number of viable units per milliliter, as determined by plate counting on oleic acid-albumin solid medium, was 20×10^6. For macrophage infection the content of one vial was rapidly thawed and appropriate dilutions were made in NCTC-109 (Microbiological Associates, Bethesda, Md.).

Immunization of mice. Male CF-1 mice (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 14 to 16 g were injected intraperitoneally with 1.0 mg (approximately 10^6 viable units) of BCG suspended in 0.2 ml of saline. Control mice received 0.2 ml of saline. Four to 6 weeks after immunization, the spleens of immunized and control mice were removed aseptically and their lymphocytes were isolated.

Isolation of splenic lymphocytes. The technique of Mishell and Dutton (15) was used for the isolation of splenic lymphocytes. The adherent cells (macrophages) were eliminated by incubating suspensions of splenic cells in plastic petri dishes for 1 h at 37°C in humidified air containing 5% CO_2. Nonadherent cells, hereafter referred to as sensitized and normal lymphocytes, were counted in a hemocytometer. They were adjusted to the desired concentration in NCTC-109 before being added to cultures of H37Rv-infected macrophages (see below). In other experiments, sensitized and normal lymphocytes were disrupted in order to isolate and characterize the mycobacterial growth inhibitory factor.

Preparation of lymphocyte extracts. Packed lymphocytes (sensitized and normal) were first treated with 1 volume of distilled water for 30 s to hemolyse the contaminating mouse erythrocytes. The reaction was stopped by adding an equal volume of an NaCl solution (0.30 M). Treated cell suspensions were centrifuged at $200 \times g$ for 10 min, and lymphocytes were then washed three times with isotonic saline. After the last washing, lymphocytes were suspended in distilled water and submitted to 10 cycles of alternate freezing and thawing using a dry ice-acetone bath and a 37°C water bath. Cellular debris were removed by centrifugation at $360,000 \times g$ for 3 h at 4°C. The supernatant, hereafter referred to as lymphocyte extract, was stored at −20°C until used. In other experiments, lymphocyte extracts were dialyzed against distilled water, and both the dialyzable and nondialyzable portions were lyophilized.

Characterization of the MycoIF. (i) Gel filtration on Sephadex G-200. The lyophilized, nondialyzable portion of lymphocyte extracts was fractionated on a column (2.5 by 40 cm) of Sephadex G-200 at room temperature. The void volume of such a column, as calibrated with blue dextran 2000, was 38 ml. Twenty milligrams (on a dry weight basis) of samples, dissolved in 2 ml of phosphate-buffered saline (0.15 M), pH 7.3, was applied on top of the column and eluted with phosphate-buffered saline at a flow rate of 30 ml/h. The absorbancy of the eluate was measured at 280 nm. The chromatogram was divided into two fractions corresponding to the void volume of the column and the lower-molecular-weight materials, respectively. After dialysis against distilled water, both fractions were lyophilized.

(ii) Treatment with ribonuclease and deoxyribonuclease. Bovine pancreatic ribonuclease (5× crystallized; Nutritional Biochemicals Corp.) was first solubilized in phosphate buffer (0.15 M, pH 7.4) and added to lymphocyte extracts in a ratio of 0.05 mg of enzyme per mg of lyophilized extracts. Deoxyribonuclease (2× crystallized; Nutritional Biochemicals Corp.) was solubilized in phosphate buffer containing 0.1 M $MgSO_4$ and added in a ratio of 0.01 mg of enzyme per mg of lyophilized extracts. Both preparations were incubated at 4°C for 18 h and at 37°C for 1 h. After dialysis against distilled water the preparations were lyophilized.

(iii) Tuberculin activity. A group of 15 CF-1 mice was sensitized with BCG (1.0 mg intraperitoneally). Four weeks later, each animal was injected with whole extract prepared from 1.1×10^7 immune spleen lymphocytes in the right hind footpad and with a corresponding extract prepared form normal splenocytes in the left hind footpad. Readings were done 24 and 48 h later by determining with dial gauge calipers the thickness of both feet and calculating the difference.

(iv) Protection of H37Rv-infected mice. Groups of H37Rv-infected mice were treated with water-soluble extracts of sensitized and normal spleen lymphocytes to see whether the MycoIF could exert some effects in vivo. In the first experiment, one dose of lymphocyte extracts, corresponding to 5×10^7 spleen lymphocytes, was given intraperitoneally to each mouse at the time of the intravenous infection with 300,000 viable units of H37Rv. In a second experiment, the same dose was given 1 day before, the same day as, and 1 day after the day of infection. Three weeks later the mice were sacrified, and the extent of the pulmonary infection was evaluated by the lung density technique of Crowle (6), as used by Portelance et al. (19) in our Institute.

(v) Effect on BCG grown in vitro. Soluble extracts prepared from 4×10^5 normal and sensitized lymphocytes were added per milliliter of Sauton medium. BCG was cultured at the surface of that medium during 14 days at 37°C, after which the yield (dry weight) of bacilli was determined.

Assay for mycobacterial growth inhibitory activity. (i) Preparation of peritoneal macrophage cultures. Normal CF-1 mice were killed by cervical dislocation, and their noninduced peritoneal cells

were collected in physiological saline supplemented with 5 U of heparin sodium per ml. After three washings in saline lacking heparin, the cells of individual mice were pooled, suspended in NCTC-109 containing 50 U each of penicillin and nystatin per ml, and counted in a hemocytometer. Viability of cells was greater than 90%, as judged by exclusion of 0.1% trypan blue. The cellular concentration was adjusted to 5×10^6 cells/ml in NCTC-109, and 0.2 ml of the suspension was layered on sterile cover slips (11 by 22 mm) placed in 35-mm-diameter plastic tissue culture dishes. After incubation for 1 h at 37°C in a 5% CO_2 incubator, nonadherent peritoneal cells were washed away with saline and the adherent cells (approximately 10^6/cover slip) were fed with 1 ml of fresh culture medium (55% NCTC-109, 40% horse serum, 5% of a 1:5 dilution of bovine embryo extract) containing the above-mentioned antibiotics. The macrophage cultures were then replaced in the CO_2 incubator for 48 h.

(ii) Infection of macrophage monolayers. The method of Patterson and Youmans (18) was used for the infection of macrophages with the H37Rv strain of *M. tuberculosis*. Briefly, 1 ml of dispersed bacilli suspended in culture medium (75% NCTC-109, 25% horse serum) and corresponding to a ratio of 2 to 3 viable units/macrophage was added to each culture dish. After 1 h of incubation at 37°C in a CO_2 incubator, nonphagocytized bacilli were removed by three washings with saline. Under these conditions, from 15 to 25% of the macrophages were parasitized, with a mean of two to three bacilli per infected cell. Higher number of bacilli per macrophage were found to lead to a higher percentage of infected cells and a higher number of intracellular bacilli, but to a more rapid lysis of macrophage monolayers.

(iii) Addition of viable lymphocytes or cell-free extracts to macrophage cultures. On the day of macrophage infection and 3 and 6 days later, viable splenic lymphocytes from normal and BCG-immunized mice, or water-soluble extracts prepared from these cells, were added to the cultures of normal macrophages infected with H37Rv. Unless otherwise stated, a concentration of 5×10^7 lymphocytes (normal or immune) or the extracts derived from a corresponding number of cells, respectively suspended or dissolved in a 1-ml volume of culture medium, was used in each culture dish. This concentration of lymphoid cells was found by Patterson and Youmans (17) and in our preliminary experiments to give a highly significant reduction in the rate of growth of intracellular mycobacteria. Every other day, when no lymphocyte preparations were added, each culture dish was fed with 0.5 ml of fresh culture medium without removing the old medium.

In a few experiments the effect of viable lymphocytes and their soluble extracts on the uptake of mycobacteria by macrophage monolayers was investigated. Thus, extracts from, or 5×10^7, lymphocytes were added simultaneously to macrophage cultures with the bacilli instead of 1 h later. After a 1-h incubation, macrophage monolayers were washed with NCTC-109 and the cover slips were mounted for microscopic examination.

Evaluation of mycobacterial growth inhibitory activity. At days 6 and 9 of macrophage cultures, that is, after the addition of two and three doses of lymphocyte preparations, respectively, three cover slips corresponding to each macrophage culture were removed from the dishes, fixed in Carnoy solution, stained according to the procedure of Ziehl-Neelsen, and then counterstained with Giemsa. After air drying, the cover slips were mounted in Permount for microscopic examination.

To evaluate the results, the number of intracellular bacilli in 100 intact infected macrophages, chosen at random in each cover slip, was determined. A value of 200 bacilli was given, as determined by Patterson and Youmans (18), when, at days 6 and 9 of culture, the number of bacilli in a given macrophage was too numerous to count. The mean value was calculated from the data of the three cover slips. The percentage of inhibition of the intracellular growth of H37Rv was calculated according to the following formula: percentage of inhibition = [100 − (mean number of bacilli per infected macrophage cultured in the presence of sensitized lymphocytes or extracts/mean number of bacilli per infected macrophage cultured in the presence of normal lymphocytes or extracts)] × 100.

In some experiments, the percentage of infection, that is, the ratio of infected macrophages over noninfected macrophages multiplied per 100 using 10 randomly chosen microscopic fields per cover slip, was calculated.

RESULTS

A typical experiment illustrating the activity of viable spleen lymphocytes from BCG-immunized mice on the intracellular proliferation of the H37Rv strain of *M. tuberculosis* within normal peritoneal macrophages is shown in Fig. 1. As seen, sensitized lymphocytes had a marked inhibiting effect on the growth on these virulent bacilli, the percentage of inhibition being 79.3 and 83.1, respectively, at days 6 and 9 after macrophage infection when compared with normal lymphocytes. In this experiment, lymphocytes from nonimmunized mice had no inhibiting effect. However, quite often we have observed that normal spleen lymphocytes, in particular those isolated from old mice (e.g., 8 to 10 months) or from groups of mice with skin lesions resulting from mice biting, had a detectable inhibiting effect, but this degree of inhibition was always lower than that of lymphocytes from BCG-immunized mice.

In another series of experiments, we have compared the activity of viable lymphocytes, hydrosoluble whole lymphocyte extracts, and the dialyzable and nondialyzable portions of these extracts on the intramacrophage proliferation of H37Rv. It should be noted that, in the experiments presented in Table 1, all tested preparations were derived from similar num-

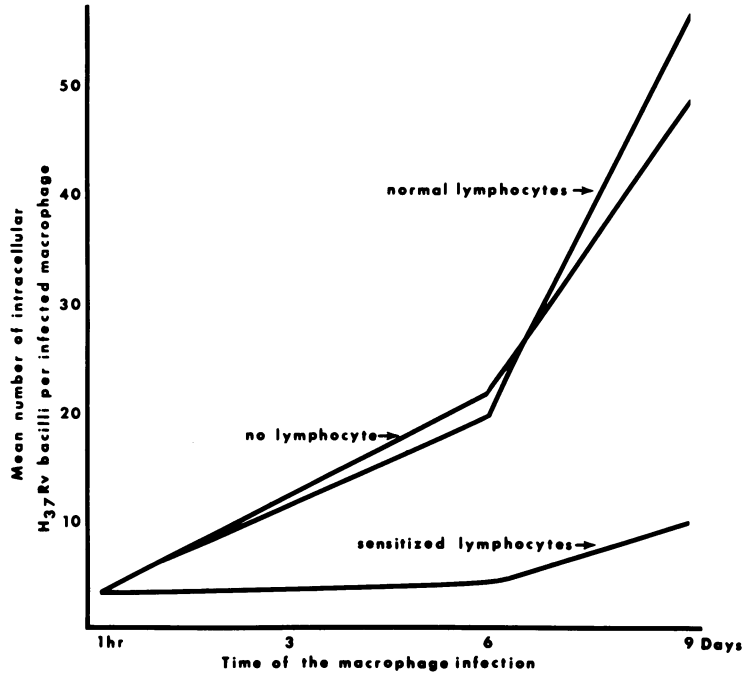

FIG. 1. *Activity of normal and sensitized viable lymphocytes on the intracellular proliferation of virulent tubercle bacilli within normal peritoneal macrophages in vitro.*

TABLE 1. *Mycobacterial inhibiting activity of sensitized viable lymphocytes, whole soluble lymphocyte extracts, and dialyzable and nondialyzable extracts measured 6 and 9 days after infection of macrophages with the H37Rv strain of M. tuberculosis*

Prepn[a]	Mouse	At day 6		At day 9	
		Mean no. of H37Rv/infected cell[b]	% Inhibition	Mean no. of H37Rv/infected cell[b]	% Inhibition
Viable lymphocytes	Normal	15.0		54.4	
	Sensitized	4.0	73.4	8.2	84.9
Whole lymphocyte extracts	Normal	18.4		60.6	
	Sensitized	6.8	63.1	19.8	67.4
Nondialyzable extracts	Normal	14.1		52.4	
	Sensitized	8.8	37.6	18.1	65.5
Dialyzable extracts	Normal	13.1		47.0	
	Sensitized	14.6	−11.5	50.1	−6.6

[a] 5×10^7 viable lymphocytes or extracts prepared from a corresponding number of cells were added to the culture of infected macrophages at 1 h and 3 and 6 days after infection.

[b] Mean number of three cover slips (100 infected macrophages per cover slip).

bers of sensitized or normal spleen lymphocytes, that is, 5×10^7 cells. It is seen that whole lymphocyte extracts of immunized mice possessed significant mycobacterial inhibiting activity, the percentage of inhibition being 63.1 and 67.4 at days 6 and 9, respectively. These data were slightly less than those obtained with viable lymphocytes, which gave 73.4 and 84.9% inhibition for the same periods of observation.

Table 1 also shows that the MycoIF was detected in the nondialyzable portion of sensitized lymphocyte extracts, whereas the corresponding dialyzable portion was completely devoid of such activity. Here, again, the inhibiting activ-

ity was slightly reduced as compared with whole extracts. However, when the number of lymphocytes was doubled (10^8) to prepare the same extract, its inhibiting activity was comparable to that of the 5×10^7 viable sensitized lymphocytes (see Table 2).

Dialyzable portions of sensitized and normal lymphocyte extracts were fractionated on Sephadex G-200. A typical chromatogram obtained with sensitized lymphocyte extracts is shown in Fig. 2. Two main fractions were obtained; fraction I, being the smallest, was eluted in the void volume of the column, whereas the bulk of the absorbing material was eluted in fraction II. Extracts prepared from normal lymphocytes led to essentially similar results. The mycobacterial inhibiting activity of these fractions from sensitized lymphocyte extracts is shown in Table 2. Corresponding fractions obtained from normal lymphocytes served as controls in these experiments. As seen, all the inhibiting activity was present in fraction I.

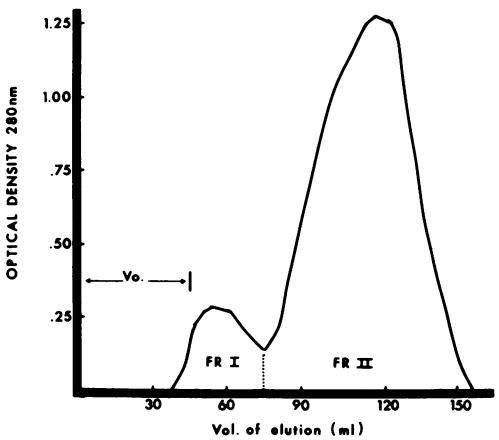

FIG. 2. *Fractionation by column chromatography on Sephadex G-200 of lymphocyte extracts from BCG-sensitized mice. (Vo indicates the void volume of the column as calibrated with blue dextran 2000).*

Further characterization of the MycoIF revealed that it was not inactivated by the enzymes deoxyribonuclease and ribonuclease. Preliminary experiments indicate that it is not completely destroyed by trypsin. Moreover, lymphocyte extracts stored at −20°C for a period of 3 months have been found to retain antimycobacterial activity. The growth-inhibiting activity of sensitized lymphocyte extracts was not abolished by heating at 56°C for 30 min but was destroyed by heating at 100°C for 10 min.

In a last series of experiments, we have investigated whether the MycoIF could have some direct effects in vivo. To eliminate the possible presence of mycobacterial antigens that might activate macrophage in vitro, extracts prepared from sensitized spleen lymphocytes were used as skin test antigens in the footpads of BCG-immunized mice. None of these mice gave a positive footpad test, whereas, under the same experimental conditions, control sensitized mice reacted positively to 10 μg of tuberculin purified protein derivative.

Attempts to demonstrate the presence of skin reactive factor in sensitized lymphocyte extracts by provoking a positive tuberculin-type reaction in the footpads of normal mice have failed.

Results of experiments designed to investigate whether the MycoIF could affect the evolution of pulmonary tuberculosis in mice are presented in Table 3. As seen, mice inoculated with three doses of sensitized lymphocyte extracts were protected against the disease as compared with saline-treated mice ($P < 0.02$).

TABLE 2. *Mycobacterial inhibiting activity of lymphocyte fractions obtained by column chromatography on Sephadex G-200*

Prepn[a]	Mouse	At day 6		At day 9	
		Mean no. of RH37Rv/infected cell[b]	% Inhibition	Mean no. of H37Rv/infected cell[b]	% Inhibition
Whole lymphocyte extracts	Normal	19.6		55.7	
	Sensitized	4.5	77.1	10.1	81.9
Fraction I	Normal	18.2		62.9	
	Sensitized	5.1	72.0	24.3	61.4
Fraction II	Normal	23.6		48.8	
	Sensitized	21.5	8.9	52.3	−7.1

[a] Extracts or fractions prepared from 10^8 lymphocytes were added to cultures of infected macrophages.
[b] Mean number of three cover slips (100 infected macrophages per cover slip).

TABLE 3. *Influence of sensitized and normal lymphocyte extracts on pulmonary tuberculosis in mice*

Mice treated with:	Mean lung density[a]	*P* value[b]
Sensitized lymphocyte extracts[c]	0.858 ± 0.041	<0.02
Normal lymphocyte extracts[c]	0.873 ± 0.056	0.1
Saline	0.901 ± 0.032	
BCG[d]	0.766 ± 0.038	
Control[e]	0.710 ± 0.031	

[a] Mean density ± standard deviation; 15 mice per group.

[b] When compared to saline-treated mice.

[c] Each mouse received three doses of lymphocyte extracts: 1 day before, the same day, and 1 day after the challenge with 300,000 viable units of H37Rv.

[d] BCG was given 3 weeks before the challenge with H37Rv.

[e] Untreated mice not challenged with H37Rv.

On the other hand, normal lymphocyte extracts also gave some degree of protection, but this was not statistically different when compared with saline-treated or sensitized lymphocyte extract-treated mice. By comparison with the protection conferred by BCG vaccine, it is seen that sensitized lymphocyte extracts had lower protecting activity. No significant protection was obtained in a first experiment when one dose (instead of three) of sensitized cell extracts was used.

Finally, cell extracts prepared from sensitized spleen lymphocytes were incorporated in Sauton medium to investigate their possible inhibiting effect on the in vitro growth of BCG. No inhibition was observed, since after 14 days of incubation the yields of dry bacilli per milliliter of Sauton medium were 29.4, 31.4, and 30.6 mg for media containing, respectively, sensitized extracts, normal extracts, and no extract.

In all of the above experiments dealing with the inhibition of intracellular bacterial growth, no significant difference has been observed between the percentage of infected macrophages in cultures treated with sensitized and normal lymphocytes (viable cells or cellular extracts). For example, in five consecutive experiments, the average percentage of infected macrophages before the addition of lymphocyte extracts was 22.3; at day 6 the mean percentages were 21.1, 20.0, and 23.8 for macrophage monolayers cultured without, with normal, and with sensitized lymphocyte extracts, respectively; and at day 9 these values increased to 42.6, 36.6, and 38.7%. In contrast, when viable lymphocytes or lymphocyte extracts were added to macrophage monolayers simultaneously with the tubercle bacilli (instead of being added 1 h after infection), a large number of cells were found to phagocytose. This observation is illustrated in Table 4. As seen, the mean number of bacilli per infected macrophage was not influenced by the kind of lymphocyte preparations added, whereas a large number of normal macrophages had phagocytosed mycobacteria when treated with the sensitized preparations. Moreover, in contrast to the MycoIF, which was not dialyzable (Table 1), the soluble factor responsible for the stimulation of the phagocytic activity was detected in both the dialyzable and nondialyzable fractions. These results cannot be explained by the fact that sensitized lymphocytes could be more toxic for macrophages than nonsensitized lymphocytes, since the total number of macrophages per cover slip after the 1-h incubation period was similar with both preparations.

DISCUSSION

The results described above reveal that spleen lymphocytes from BCG-immunized mice have the capacity to inhibit the proliferation of the H37Rv strain of tubercle bacilli within normal peritoneal macrophages in vitro, thus confirming the previous observations of Patterson and Youmans (17) and of others working with unrelated intracellular pathogens (9, 13, 21). Generally speaking, spleen lymphocytes from nonimmunized mice were also found to inhibit,

TABLE 4. *Phagocytic activity of normal macrophages after 1 h of incubation with tubercle bacilli in the presence of viable lymphocytes and soluble lymphocyte fractions*

Prepn[a]	Mouse	Mean no. of H37Rv/infected cell[b]	% Infected macrophages[c]
Viable lymphocytes	Normal	2.5	17.7
	Sensitized	2.3	43.6
Nondialyzable fraction	Normal	2.7	22.3
	Sensitized	2.8	38.2
Dialyzable fraction	Normal	2.2	18.9
	Sensitized	2.7	41.9
Culture medium[d]		2.3	23.7

[a] 5×10^7 viable lymphocytes or fractions corresponding to a similar number of cells were used in each culture dish.

[b] Mean number of three cover slips (100 infected macrophages per cover slip).

[c] Number of infected macrophages over number of noninfected macrophages × 100 in 10 randomly chosen microscopic fields per cover slip.

[d] 55% NCTC-109, 40% horse serum, and 5% of a 1:5 dilution of bovine embryo extract.

but to a smaller extent, the intracellular growth of H37Rv. Many factors, such as the mouse strain, the number of lymphocytes added to macrophage monolayers (12, 17), and, as observed in this study, the age and the clinical state of the animal, would be responsible for this low inhibiting activity. An alternative explanation could reside in the fact that, as shown by Klun and Youmans (11), the presence of extracellular tubercle bacilli in macrophage cultures can induce normal lymphocytes to form and release small amounts of MycoIF. In fact, such a situation possibly occurred in the present study, because at days 6 and 9 of macrophage cultures we observed that approximately 5% of the macrophages (in particular, the heavily infected ones) were partially destroyed, liberating their bacterial content into the medium. Nevertheless, since in the present work the antimycobacterial activity of sensitized lymphocytes was compared with that of nonsensitized lymphocytes, the interpretation of our results was facilitated and remained valid.

Most previous investigators have studied the bacterial inhibiting activity of either viable lymphocytes or cell-free supernatants from antigenically stimulated lymphocytes. In contrast, the present work shows that a MycoIF can be extracted directly from sensitized lymphocytes without the need of incubating them with the specific antigen. This finding is direct evidence that a MycoIF is already present in vivo in lymphocytes specifically sensitized to BCG. The fact that sensitized lymphocytes contain in vivo an antimycobacterial substance would not be surprising when one considers that BCG can develop and thus persist for several weeks in the organs (spleen and liver) of infected mice (4, 5). Consequently, since in the present study spleen lymphocytes were isolated 4 to 6 weeks after immunization, the presence of BCG in the spleen presumably might still stimulate lymphocytes to produce the MycoIF.

On the basis of corresponding number of cells, soluble lymphocyte extracts had less inhibiting activity than viable sensitized lymphocytes (Table 1). However, when the number of lymphocytes used for extractions was doubled, the inhibiting activity increased to the level of that obtained with viable lymphocytes. These results would suggest that some inhibiting activity was lost during the extraction of the factor. Unfortunately, the insoluble portion of the lymphocyte extracts has not been tested, in order to eliminate completely the presence of some inhibiting activity in this fraction. An alternate explanation for the higher activity of viable lymphocytes would be the presence of extracellular tubercle bacilli in macrophage cultures, which, as discussed above, could induce sensitized lymphocytes to continue to form and release the MycoIF. Such a situation presumably does not occur when cell-free extracts are used in macrophage cultures.

The present study has established that the lymphocyte MycoIF was not dialyzable and was unaffected by deoxyribonuclease and ribonuclease, lyophilization, and storage at −20°C for long periods of time but was inactivated by heat (100°C for 10 min). All these characteristics corroborate those reported recently by Cahall and Youmans (1, 2) for the MycoIF present in cell-free supernatants of antigenically stimulated lymphocytes. In contrast, these workers found that the molecular weight of their antimycobacterial substance was between 20,000 and 35,000 on the basis of protein standards filtered through Sephadex G-150 (2), whereas our results suggest that the molecular weight of the active substance was much higher since it was eluted in the void volume of Sephadex G-200 columns. Although presently unknown, the reasons for these divergent results might be explained by the fact that both antimycobacterial factors were obtained by two different methods. Obviously, other physicochemical characteristics, such as sensitivity to neuraminidase, buoyant density, etc. (16), of our antimycobacterial factor would be needed before attempting to relate it with the MycoIF of Cahall and Youmans and with one of the recognized mediators of cellular hypersensitivity known to affect macrophages (e.g., macrophage activating factor, macrophage migration inhibitory factor, etc.).

Although soluble extracts from sensitized lymphocytes inhibit the intracellular growth of tubercle bacilli, treatment of H37Rv-infected mice with these extracts led to negligible protecting effects, in particular when compared with the results obtained in mice treated with normal lymphocyte extracts. This failure to confer more significant protection against tuberculosis might be due to the fact that the experimental conditions and the method of evaluation as used in this study were not the most appropriate ones. However, in relation to these nonconclusive results, it should be mentioned that Mackaness (14) has reported that resistance to *Listeria monocytogenes* can be successively tranferred in mice only when the sensitized lymphoid cells are viable. Similarly, the water-soluble constituents of sensitized lymphocytes were without any detectable effect on the growth of BCG on Sauton medium. This observation, which confirms the previous re-

sults of Klun and Youmans (11), strongly suggests that the MycoIF cannot exert a direct effect in vitro on mycobacteria.

Finally, this study has shown that sensitized lymphocytes contained other soluble factors that, when added simultaneously with suspensions of tubercle bacilli to macrophage monolayers, promote the phagocytic activity of these cells. It is interesting to note that Svejcar et al. (23) have also observed similar stimulating effects when using cell-free supernatants obtained by exposing lymphocytes of BCG-immunized rabbits to tuberculin purified protein derivative. Although preliminary, our results indicate that possibly two phagocytosis-promoting factors exist, because both the dialyzable and the nondialyzable portion of sensitized lymphocyte extracts were active. Since one of these factors is dialyzable, it can thus be distinguished from the MycoIF. Our results suggest also that these factors do not induce macrophages to phagocytose large amounts of bacilli but stimulate a greater number of normal macrophages to phagocytose. It is possible that the nondialyzable phagocytosis-promoting factor could be identified as cytophilic antibodies. In fact, one of us has already shown that "cell-fixed" hemagglutinating mycobacterial antibodies are present in leukocyte extracts of tuberculin-sensitive patients (8).

The exact role played by both MycoIF and phagocytosis-promoting factors in host defense against tuberculosis is not known.

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