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Mechanisms of Action of *Mycobacterium bovis* BCG-Induced Suppressor Cells in Mitogen-Induced Blastogenesis

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Spleen cells from *Mycobacterium bovis* BCG-infected C57Bl/6 mice when cultured in vitro for 72 h elicited soluble suppressor mediators capable of nonspecifically suppressing the mitogen-induced blastogenesis of normal splenocytes. Maximal production of suppressor mediators occurred during the first 24 h in culture, and their production ceased after 72 h. Attempts to isolate the mediators from fresh nonincubated splenocytes failed. After incubation, a strong residual suppressive activity was constantly detected in cell preparations used for production of suppressor factors. Supernatants prepared from cultures of spleen cells of mice infected 14 days earlier possessed higher suppressive activity than did those obtained 28 days after infection. In contrast, the residual cellular suppressive activity increased during the course of the infection. Although the activity of soluble factors was not inhibited, the residual activity of incubated cells was highly depressed by the presence of mouse erythrocytes in the cultures. Thus, the incubated cells appear to act through a direct cell-to-cell contact with the mitogen-responding cells. Finally, the results of depletion experiments suggest that the two populations of BCG-induced suppressor cells, namely, T lymphocytes and macrophage-like cells, are able to elicit suppressor mediators and to retain thereafter suppressive activity.

Mice infected with various species of mycobacteria have been shown to have splenic suppressor cells which can inhibit the proliferative responses to polyclonal mitogens, alloantigens, and specific antigens (15, 24, 25) and other in vitro immune functions such as the generation of cytotoxic response (20), antibody synthesis (8, 11), and the expression of natural killing activity (17). The exact nature of mycobacteria-induced suppressor cells is still a matter of controversy. Several investigators have identified them as macrophage-like cells (1, 20) or as macrophage-granulocyte precursor stem cells (5); others have felt that they are T lymphocytes (10, 24). Evidence that these two suppressive populations coexist in the spleens of infected mice has also been presented (8, 15, 26). The mechanism of action of mycobacteria-induced suppressor cells has not been completely elucidated. Bullock et al. (8) reported that the spleen cells from *Mycobacterium lepraemurium*-infected mice elaborated a soluble suppressor factor(s) that depressed the plaque-forming cell response of normal spleen cells to sheep erythrocytes. Since they worked with unseparated spleen cells, it is not known which of the two cell types (macrophages or T lymphocytes) was generating the factor(s). Klimpel (19) showed that although *M. bovis* BCG-induced macrophage-like suppressor cells inhibited both in vitro plaque-forming cells and cytotoxic responses, only the suppression of the antibody response was mediated by a soluble factor(s). On the other hand, Brown et al. (7) reported that intact spleen cells from BCG-infected mice were required to suppress the in vitro plaque-forming cell response of normal spleen cells.

Having observed recently that macrophage-like and T-suppressor cells coexisted in the spleens of BCG-infected mice and that both were involved in the inhibition of the mitogen-induced lymphoproliferation of normal spleen cells (26), we wished to investigate the mechanisms of action of each on this in vitro cellular response. Our results indicate that both types of suppressor cells are able to elaborate soluble suppressor mediators in culture. Moreover, after the elaboration of soluble factor, the cells maintain a strong suppressive activity, and they appear to act through a direct cellular contact with the mitogen-responding cells.

MATERIALS AND METHODS

Mice. Female C57Bl/6 mice, 2 to 3 months old, obtained from The Jackson Laboratory, Bar Harbor, Maine, were used throughout.

BCG. Mice were injected intravenously with 1 mg (about 10^7 viable units) of a lyophilized preparation of

the Montreal substrain of *M. bovis* BCG (Institut Armand-Frappier, Laval-des-Rapides, Quebec).

Spleen cell suspensions. Mice were sacrificed by cervical dislocation 14 to 28 days after infection. Pooled spleen cells from two to three normal or BCG-infected mice were used in most experiments. They will be referred to as normal and BCG spleen cells, respectively. After elimination of contaminating erythrocytes by osmotic shock, spleen cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (all reagents were obtained from GIBCO Laboratories, Grand Island, N.Y.).

Supernatants. Normal and BCG spleen cells were distributed in flat-bottomed tissue culture plates (2.5×10^5 cells in 0.1 ml/well) (Linbro Scientific, Hamden, Conn.) and incubated in a humidified atmosphere containing 5% CO_2 for 72 h at 37°C. Cells were then pelleted by centrifugation at 800 × *g* for 10 min, and supernatants from each group were collected, centrifuged at 10,000 × *g* for 20 min, and filtered through a membrane filter (0.22-μm pore size). Remaining cells and debris were washed twice in the culture plates with 0.3 ml of the culture medium, and 2.5×10^5 fresh normal spleen cells in 0.1 ml were then added to each well. Fresh normal spleen cells were also mixed in equal volume with supernatants and redistributed in culture plates at a concentration of 2.5×10^5 cells/well. Thereafter, these cellular preparations were incubated in the presence of mitogens and tested for thymidine incorporation.

Elimination of suppressor cells. Suppressor T lymphocytes were lysed by using an anti-Thy 1 antiserum (Cederlane Laboratories Ltd., Hornby, Ontario) and complement; macrophage-like suppressor cells were removed magnetically after carbonyl iron treatment under conditions previously described (26). After treatment, cells were washed two to three times in culture medium, counted for viability in a hemacytometer, adjusted to the desired concentration, and incubated for the production of supernatants. Under our conditions, 94 to 98% of thymocytes and 33 to 35% of splenocytes were lysed by the antiserum, and about 1% of the nonphagocytic cells were stained for nonspecific esterases.

Lymphoblastic transformation. The mitogenic responses of fresh normal spleen cells in admixture with supernatants or preincubated spleen cells were determined by measuring the incorporation of tritiated thymidine ([^{3}H]TdR) into DNA according to the experimental conditions already published (27). Briefly, viable nucleated cells were cultured with optimal concentrations of mitogens, i.e., 7.5 μg for phytohemagglutinin (PHA; GIBCO), 1.0 μg for concanavalin A (ConA; Calbiochem, La Jolla, Calif.), and 5.0 μg for lipopolysaccharide (LPS O26:B6; Difco Laboratories, Detroit, Mich.) per well. All cultures were set up in triplicate in flat-bottomed tissue culture plates and incubated for 72 h at 37°C in a humidified atmosphere containing 5% CO_2. Cultures were pulsed with 1.0 μCi of [^{3}H]TdR 18 h before the end of the incubation period and harvested on glass fiber filters with a Titertek cell harvester (Flow Laboratories, Inc., Mississauga, Ontario). The radioactivity was counted in a Beckman DPM-100 scintillation spectrometer. The results were expressed as the mean net thymidine incorporation, (i.e., mean counts per minute of the triplicate stimulated cultures minus the mean counts per minute of the triplicate nonstimulated cultures). The percentage of suppression was calculated as follows:

$$\text{Percentage of suppression} = \left(1 - \frac{\text{cpm}^{\text{BCG}}}{\text{cpm}^{\text{normal}}}\right) \times 100\%$$

where cpm^{BCG} and $\text{cpm}^{\text{normal}}$ represent the mean net incorporation by the fresh normal spleen cells in the presence of supernatants or preincubated cells, respectively, from BCG-treated and from normal mice.

In some experiments, various concentrations of mouse erythrocytes (MRBC) were added during the lymphoblastic transformation assay. Heparinized blood was obtained by cardiac puncture from normal C57Bl/6 mice. Blood cells were washed three times in culture medium, counted, and adjusted to the desired concentrations.

RESULTS

Inhibiting activity of supernatants and preincubated BCG spleen cells. Unseparated spleen cells from normal and BCG-infected mice were first cultured for 72 h; then the supernatants and the corresponding preincubated cells (i.e., those from which the supernatants derived) were tested for suppression of the mitogenic response of normal splenocytes. Table 1 shows the mean data obtained in five independent experiments performed at various times (from 14 to 28 days) after infection. Supernatants obtained from normal spleen cell cultures did not depress the responses of normal splenocytes to PHA and ConA, but they slightly depressed their response to LPS. In contrast, supernatants from BCG spleen cells markedly inhibited the incorporation of thymidine into the splenocytes regardless of the mitogen used. Moreover, the preincubated BCG spleen cells still exhibited an elevated inhibitory activity, whereas the preincubated normal spleen cells did not interfere with the mitogenic response of splenocytes. Thus, BCG spleen cells could release soluble suppressor mediators in cultures and, in addition, retain their suppressive activity after the 72-h incubation.

The inhibitory activity of supernatants (and of preincubated cells) was directly related to the concentration used in the cultures. Filtration of supernatants through a 0.22-μm membrane filter (see Materials and Methods), which completely eliminated all free acid-fast bacilli, did not modify their suppressive activity, showing that BCG present in the cultures was not responsible for the suppression observed. Intracellular and extracellular acid-fast bacilli were constantly observed in the preincubated cells, but the num-

TABLE 1. Effects of supernatants and corresponding preincubated cells from normal and BCG-infected mice on mitogenic responses of normal splenocytes

Constituent added to normal splenocytes[a]	Net [^{3}H]TdR incorporation[b] after stimulation with:		
	PHA	ConA	LPS
Fresh culture medium	22.9 ± 6.3	117.2 ± 13.9	38.3 ± 6.3
Supernatant from normal spleen cells	33.1 ± 5.7	114.9 ± 17.4	20.8 ± 2.8
Preincubated normal spleen cells	24.1 ± 4.0	108.5 ± 8.4	32.4 ± 8.8
Supernatant from BCG spleen cells	13.0 ± 5.2	57.8 ± 17.3	7.6 ± 2.1
Preincubated BCG spleen cells	17.1 ± 2.0	30.4 ± 27.7	14.0 ± 13.7

[a] Supernatants and preincubated cells prepared from 2.5×10^5 normal or BCG spleen cells after 72-h cultures were added to 2.5×10^5 fresh normal splenocytes.

[b] Difference in counts between mitogen-stimulated and nonstimulated cultures, expressed as counts per minute $\times 10^{-3}$ of triplicate cultures from five separate experiments ± standard error of the mean.

ber of bacilli detected in these preparations (about 4.75×10^3 per 2.5×10^5 incubated cells) appeared too low to account for the reduction of thymidine incorporation. In fact, a study of the dose-response effect of BCG added to cultures of 2.5×10^5 normal spleen cells revealed that 5×10^6 bacilli optimally stimulated thymidine incorporation, whereas at higher doses suppression of thymidine incorporation occurred (S. Lemieux and R. Turcotte, unpublished data).

Kinetics of production of soluble suppressor mediators in culture. Supernatants from BCG spleen cell cultures were prepared as usual (see Materials and Methods) and tested for suppression after 24, 48, and 72 h of incubation. A significant suppressive activity was readily detectable at 24 h; it increased till 48 h and then remained more or less at the same level until 72 h. To determine more precisely when suppressor mediators ceased to be produced and to eliminate the possible detrimental effect of nutrient depletion on their production, BCG spleen cells were incubated in a culture tube at a concentration of 5×10^6 cells/ml for 96 h, and culture medium was replaced daily after supernatant collection. Suppressor activity was tested by adding 0.1-ml portions of the supernatants collected from each 24-h period to the culture of fresh splenocytes (5×10^5 cells/ml) before mitogen stimulation. The suppression of ConA-induced mitogenic response was optimal in supernatants from the first 24 h and decreased thereafter to reach a negligible level by 72 h, indicating that the production or the release of suppressor mediators occurred early during the incubation period (Table 2). Similar results were obtained when spleen cells were stimulated with PHA or LPS. These experiments also showed that supernatants collected from the 72 h incubation period contained the optimal concentration of soluble suppressor mediators that could be produced in culture, and these conditions were used throughout this study.

To rule out the possibility that cytotoxic factors liberated from dead cells during the incubation period were responsible for the suppression, soluble extracts from fresh normal and BCG spleen cells were prepared by four cycles of freezing and thawing, and the resulting soluble constituents as well as the cellular debris were used in the lymphoblastic transformation assay (Table 3). As found previously (27), viable BCG spleen cells completely inhibited the responses of splenocytes to all three mitogens, whereas soluble and insoluble extracts from BCG spleen cells were devoid of inhibitory

TABLE 2. Kinetics of production of soluble suppressor mediators during the course of the incubation[a]

Time of incubation of BCG spleen cells (h)	[^{3}H]TdR incorporation after stimulation with ConA			
	Expt 1		Expt 2	
	Net cpm (10^3) ± SD	Suppression (%)	Net cpm (10^3) ± SD	Suppression (%)
0 (control)[b]	182.9 ± 12.1		179.3 ± 15.7	
24	138.9 ± 10.8	24.1	124.9 ± 8.7	30.4
48	170.2 ± 9.7	7.0	158.0 ± 10.4	11.9
72	185.5 ± 15.6	−1.4	181.0 ± 12.5	−0.9
96	201.9 ± 13.1	−10.3	182.5 ± 13.2	−1.7

[a] See text for details.

[b] Fresh culture medium was used.

TABLE 3. Effects of viable BCG spleen cells and their corresponding soluble and insoluble constituents on the mitogenic response of normal splenocytes

Constituent added to normal splenocytes[a]	Net [^{3}H]TdR incorporation[b] after stimulation with:		
	PHA	ConA	LPS
Viable normal spleen cells	43.8	180.8	49.0
Viable BCG spleen cells	0	0	0
Soluble extracts from normal spleen cells	36.4	202.3	51.6
Cellular debris from normal spleen cells	38.6	174.9	48.0
Soluble extracts from BCG spleen cells	40.4	158.3	46.2
Cellular debris from BCG spleen cells	43.7	167.2	50.3
Fresh culture medium	44.0	166.4	51.2

[a] 2.5×10^5 viable spleen cells or the constituents derived thereof were added to each well of tissue-culture plates containing 2.5×10^5 fresh normal splenocytes.

[b] Expressed as in Table 1. Standard deviations less than 10% were not included.

activity, indicating that soluble suppressor mediators are not preformed substances which are released into the supernatants after the lysis of spleen cells. Similarly, no suppression was observed in supernatants and preincubated cells prepared from BCG spleen cells killed by heat (56°C, 30 min) before the 72-h incubation period (data not shown).

Effect of MRBC on the suppressor mediators and preincubated cells. To investigate whether the inhibitory activity that was still present in the cells after the elaboration of suppressor mediators was due to the direct action of these incubated cells on the mitogen-responding cells or to soluble mediators either continually released by the suppressor cells during the lymphoblastic transformation assay or induced de novo in the cultures by the mitogens during this assay, we attempted to prevent the physical contact of suppressor cells with the mitogen-responding cells by adding MRBC to the cultures before mitogen stimulation. The presence of increasing amounts of MRBC in the cultures of BCG spleen cells led to a gradual restoration of the blastogenic responses to all three mitogens which peaked at ratios of 20:1 to 40:1 and decreased at higher ratios. The concentration of MRBC leading to an optimal restoration of blastogenesis in cultures of BCG spleen cells slightly depressed the incorporation of thymidine in cultures of normal spleen cell (data not shown). Table 4 shows the results obtained when MRBC were added to cultures of fresh normal splenocytes in admixture with supernatants or preincubated cells from normal and BCG mice. The blastogenic depression mediated by the supernatants from BCG spleen cells was not restored in the presence of MRBC, implying that the suppressor mediators were not neutralized or mobilized by the MRBC. In contrast, the high suppressive activity of preincubated BCG spleen cells was markedly inhibited in the presence of an optimal amount of MRBC. These cells most likely acted by preventing the physical contact of suppressor cells with accessory or mitogen-responding cells, or both.

Suppressive activity in supernatants and prein-

TABLE 4. Effects of MRBC on the inhibitory activity of supernatants and of corresponding preincubated cells from normal and BCG-infected mice[a]

Constituent added to normal splenocytes[b]	MRBC/splenocyte ratio	Net [^{3}H]TdR incorporation (10^3 cpm ± SD) after stimulation with:		
		PHA	ConA	LPS
Supernatants from:				
Normal spleen cells	0	39.2 ± 4.1	134.5 ± 5.6	17.2 ± 0.7
	4:1	31.3 ± 3.2	130.6 ± 6.3	15.5 ± 1.2
	40:1	14.3 ± 1.1	106.4 ± 6.2	13.3 ± 1.2
BCG spleen cells	0	15.8 ± 2.3	74.9 ± 2.5	8.9 ± 0.3
	4:1	12.5 ± 1.0	67.7 ± 5.2	8.5 ± 0.1
	40:1	9.6 ± 0.7	30.2 ± 3.9	6.3 ± 0.1
Preincubated cells from:				
Normal spleen cells	0	42.5 ± 4.3	164.8 ± 5.9	25.7 ± 2.2
	4:1	38.9 ± 1.4	146.0 ± 5.6	19.9 ± 2.2
	40:1	48.1 ± 2.3	184.4 ± 9.5	23.9 ± 1.6
BCG spleen cells	0	0	0	0
	4:1	0	0	0
	40:1	10.3 ± 1.1	108.9 ± 3.3	7.1 ± 0.2

[a] Cultures were made 21 days after infection.

[b] Supernatants and preincubated spleen cells obtained from 72-h cultures of 2.5×10^5 normal or BCG spleen cells were mixed with 2.5×10^5 fresh splenocytes.

TABLE 5. Inhibitory activity of supernatants and preincubated spleen cells obtained from mice at days 14, 21, and 28 after BCG infection

Constituent added to normal splenocytes[a]	[^{3}H]TdR incorporation[b] at:					
	Day 14		Day 21		Day 28	
	cpm	Suppression (%)	cpm	Suppression (%)	cpm	Suppression (%)
Supernatants from:						
Normal spleen cells	78.8 ± 16.1[b]		117.1 ± 42.5		86.4 ± 23.6	
BCG spleen cells	38.3 ± 6.6	51.4	63.8 ± 7.7	45.6	67.0 ± 5.8	22.5
Preincubated cells from:						
Normal spleen cells	94.4 ± 21.1		110.7 ± 24.5		114.0 ± 15.9	
BCG spleen cells	51.1 ± 14.5	48. 9	2.1 ± 0.9	98.1	0	100

[a] Supernatants and corresponding preincubated spleen cells obtained from 72-h cultures of 2.5×10^5 normal or BCG spleen were cultured with 2.5×10^5 fresh splenocytes in the presence of ConA.

[b] Each value represents the mean net counts per minute ($\times 10^3$) from two to three independent experiments ± standard error of the mean.

cubated spleen cells obtained from mice at different times after BCG infection. The influence of the time at which the spleen cells were cultured after infection of mice was investigated. Table 5 shows the mean inhibitory activity of supernatants and of preincubated cells obtained in two to three independent experiments, each performed at 14, 21, and 28 days after BCG inoculation. Since the results were essentially the same with all three mitogens, only those obtained with ConA are presented. At day 14, supernatants strongly inhibited the incorporation of thymidine into the mitogen-stimulated normal spleen cells. Thereafter the inhibition decreased slowly to reach a very low value at day 28. In contrast, the high inhibitory activity already present at day 14 in the preincubated cells increased further throughout the observation period. These results suggest that the suppression mediated by direct cellular contact had a longer-lasting effect than the production of soluble suppressor mediators in BCG-infected mice.

Types of cells implicated in the elaboration of suppressor mediators. Spleen cells obtained from two to three control and infected mice at 14 and 28 days after BCG inoculation were divided into three parts. The first was left unseparated, and T lymphocytes and phagocytic cells were depleted in the second and third parts, respectively, before the 72-h incubation period. Supernatants and preincubated cells obtained from these three cellular populations were tested for suppression of ConA-induced blastogenesis of normal splenocytes (Table 6). By comparison with the cultures containing supernatants and preincubated cells obtained from unseparated cellular populations, the blastogenic responses were lower in the cultures containing supernatants or preincubated cells from T-depleted populations and higher in those containing supernatants or preincubated cells from phagocyte-depleted populations. This occurred with cell preparations obtained from both normal and BCG spleen cells, except with those from preincubated BCG spleen cells 14 days after infection. These findings illustrate the well-known influence of accessory cells (most likely the macrophages) on lymphoblastic responses (4) and indicate that accessory cells can act on the mitogen-responding cells through cellular contact (18) and also via soluble mediators (9). An enrichment in lymphocytic concentration may represent an alternative explanation for the increased thymidine incorporation in the cultures containing macrophages-depleted populations. Fourteen days after infection, the supernatants and preincubated cells derived from both T-depleted and phagocyte-depleted populations of BCG spleen cells possessed inhibitory activity. Moreover, the inhibitory activity of the phagocyte-depleted preincubated cells appeared superior to that of the T-lymphocyte-depleted preincubated cells and to that of supernatants obtained from all groups. These results thus indicate that the two populations of suppressor cells which were present early after infection, as previously described (26), could elicit suppressor factors and thereafter maintain their suppressive activity. Twenty-eight days after infection, the removal of T lymphocytes before the incubation did not affect the production of the soluble mediators, and the inhibitory activity of the preincubated cells was preserved. In contrast, the removal of phagocytic cells markedly depressed the inhibitory activity in both supernatants and preincubated cells. These results, therefore, show that in the late BCG-infected mice, the suppressor macrophages acting through the production of soluble mediators and direct cellular contact were predominating.

TABLE 6. Effect of depletion of T lymphocytes and phagocytic cells on the suppressive activity of supernatants and of preincubated cells in early and late BCG-infected mice

Constituent added to normal splenocytes[a]	[^{3}H]TdR incorporation (10^3 cpm ± SD) at:			
	Day 14		Day 28	
	cpm	Suppression (%)	cpm	Suppression (%)
Supernatants from:				
Unseparated cells				
Normal	86.1 ± 0.9		47.9 ± 6.0	
BCG	45.2 ± 2.6	47.5	42.0 ± 2.9	12.4
T-depleted cells				
Normal	51.9 ± 5.2		32.4 ± 5.9	
BCG	30.2 ± 7.4	41.9	20.3 ± 1.1	37.4
Phagocyte-depleted cells				
Normal	155.2 ± 4.5		122.2 ± 9.8	
BCG	99.0 ± 6.5	36.3	133.0 ± 4.6	0
Preincubated cells from:				
Unseparated cells				
Normal	58.2 ± 7.7		93.6 ± 13.0	
BCG	18.0 ± 2.3	69.1	8.6 ± 3.6	90.9
T-depleted cells				
Normal	27.1 ± 9.8		63.1 ± 7.7	
BCG	16.0 ± 5.7	41.0	0	100
Phagocyte-depleted cells				
Normal	210.1 ± 8.9		110.0 ± 3.1	
BCG	1.5 ± 0.8	99.3	98.4 ± 9.7	10.6

[a] Supernatants and preincubated cells derived from 2.5×10^5 normal or BCG unseparated, T-lymphocyte-depleted, or phagocyte-depleted spleen cells were cultured with 2.5×10^5 fresh splenocytes stimulated with ConA.

DISCUSSION

Supernatants of spleen cell cultures from BCG-infected mice were shown to contain suppressor mediators which markedly depressed the incorporation of [^{3}H]TdR in mitogen-stimulated lymphocytes. Supernatants from normal spleen cells did not suppress the incorporation by PHA- and ConA-stimulated lymphocytes but, for still unknown reasons, they slightly depressed the incorporation by the LPS-stimulated lymphocytes (Table 1). The suppressor activity was attributable neither to the presence of free bacilli in the supernatants or in preincubated cells nor to nutrient depletion in culture medium. It also seems unlikely that cold thymidine liberated by cells dying in cultures as shown by Opitz et al. (22) can explain the inhibitory activity of supernatants, since about 80% of the suppressive activity of supernatants was found to be nondialyzable (Turcotte and Lemieux, unpublished data). All of these data clearly indicate that the BCG-induced suppressor cells can inhibit mitogen-induced blastogenesis through the elaboration of soluble suppressor factors, the nature of which remains to be established.

Of interest was the finding that the inhibitory activity decreased in the supernatants from day 14 to day 28 after BCG infection. The data obtained with cell-depleted subpopulations (Table 6) indicate that suppressor T cells were present mainly in the early stage of the infection and that the decrease in suppressive activity of supernatants at day 28 was caused by the relative lack of soluble mediators generated by the suppressor T lymphocytes.

Several other investigators have demonstrated that both suppressor T lymphocytes and macrophages (in particular activated macrophages) can generate substances inhibitory for DNA synthesis (9, 21), and many of these factors have been partially characterized (2). The biochemical nature of the factors which inhibit the in vitro plaque-forming cell response to sheep erythrocytes in the supernatants of spleen cells from BCG-infected (19) and from *M. lepraemurium*-infected (8) mice is presently unknown. Prostaglandins E (12) and immune interferon (16), which are induced after BCG inoculation (29), as well as the inhibitor of DNA synthesis (21) and the prostaglandin-induced T-cell-derived suppressor (23) should be considered as possible candidates. However, it seems unlikely that prostaglandins or the prostaglandin-induced T-cell-derived suppressor were the suppressor factors involved in our study, since we have observed recently that indomethacin, an inhibitor of prostaglandin synthesis, does not depress the suppressive activity of BCG spleen

cells in vitro. Finally, it must be emphasized that the presence during the incubation period of the specific antigen (BCG) in the cultures fulfilled all of the experimental conditions leading to the synthesis or the release of numerous lymphokines and monokines (6). As shown in the present study, the kinetics of appearance of suppressor factors in the supernatants closely paralleled that of several lymphokines, e.g., lymphotoxin (13), cloning inhibitory factor (14), and proliferation inhibitory factor (3); heat-killed, as opposed to viable, spleen cells did not elaborate suppressor mediators; and the mediators were probably not preformed entities (unless their activity was masked when complexed to the cells). All of these observations strongly suggest that the suppressor factors were induced during the incubation period.

A strong inhibitory activity was always present in the BCG spleen cells after the elaboration of suppressor mediators. To our knowledge, such a residual suppressive activity has not been described previously when cells were found implicated in the elaboration of suppressor mediators. We cannot conclude from our data whether the cells involved in the generation of soluble mediators and those with a residual suppressive activity represent the same or a different population of suppressor cells. In preliminary experiments, we observed that the soluble suppressor factors of mitogen-induced blastogenesis were without any detectable effect on the induction of cytotoxic T lymphocytes in vitro, whereas the preincubated cells did significantly depress this immune function. These data suggest that the mechanism of action of suppressor cells varies according to the nature of the immune response studied. However, that different subpopulations among each population of suppressor cells could also be implicated, as postulated by Klimpel (19), cannot be completely ruled out.

Since, as discussed above, it is possible that the soluble mediators were induced during the 72-h incubation period, the cells also could have acquired their suppressive property during this time. This would imply that spleens from BCG-infected mice contain precursor cells that differentiate into suppressor cells during the preincubation phase or during the lymphoblastic transformation assay. It seems unlikely that the suppression was induced by the polyclonal mitogens present at the time of the lymphoblastic assay; otherwise, suppression would have been detectable in cultures of mitogen-stimulated normal spleen cells.

The addition of an optimal amount of MRBC to cultures of BCG spleen cells did not interfere with the suppressive activity of soluble mediators, but the MRBC were shown to depress the activity of preincubated suppressor cells, most likely by preventing their physical contact or that of accessory cells with the mitogen-responding cells. These results confirm and extend those reported recently by Webb and Brooks (28). The inhibitory effect of MRBC when used in large amounts in the cultures could be due either to their competition for the mitogens or to their interference on lymphocyte and accessory cell contact needed for mitogen-induced blastogenesis. On the other hand, restoration of the blastogenesis in the BCG spleen cell cultures by MRBC was not found to be due to the fact that supraoptimal doses of mitogens were used and that the competition of the MRBC for the mitogens reduced the doses to an optimal level.

From this study, it can be concluded that the inhibition of blastogenesis in the spleen cells from BCG-infected mice can be mediated by two different mechanisms, soluble mediators and direct cellular contact. More recent experiments from our laboratory have indicated that these two mechanisms are also implicated in the inhibition of BCG spleen cells when stimulated in vitro with the specific antigens (i.e., tuberculin purified protein derivative) instead of the T- and B-cell mitogens. Our results illustrate the complexity of the mechanisms of action of suppressor cells and of the nature of suppressor mediators involved in the depression of thymidine incorporation by spleen cells from BCG-sensitized mice. The continued investigation of both these aspects should provide useful information about the in vitro regulation of immune response.

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