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A Role for Gamma Interferon, Tumor Necrosis Factors, and Soluble T-Cell Receptors in the Depressed Blastogenic Response of Spleen Cells of *Mycobacterium lepraemurium*-Infected Mice

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Spleen cells of *Mycobacterium lepraemurium*-infected mice were cultured on petri dishes coated with mycobacterial antigens, and antigen-reactive cells were isolated. Upon incubation in mitogen- or antigen-free culture medium, these cells released mediators capable of depressing the in vitro proliferative response of normal splenocytes to specific antigen and to concanavalin A and lipopolysaccharide. One of these mediators was identified with gamma interferon (IFN-γ), mainly on the basis that treatment of supernatants with monoclonal anti-IFN-γ antibodies markedly reduced the suppressive activity contained therein. Detectable levels of tumor necrosis factor alpha (TNF-α) and TNF-β were present in spleen cell culture supernatants of infected mice. Moreover, low doses of recombinant TNF-α and TNF-β were found to potentiate the suppressive activity of exogenous IFN-γ. Soluble T-cell receptors beta were also detected in the culture supernatants. The elimination of these molecules with monoclonal anti-T-cell receptor beta (F23.1) antibodies immobilized on a plastic surface partially reversed the depression of the response to mycobacterial antigen but did not affect the response to mitogens. These results revealed the complex nature of suppressor mediators that are produced by mycobacterial antigen-reactive cells and that regulate the in vitro proliferative response.

Mycobacterial antigen-reactive suppressor cells have recently been isolated from the spleens of mice infected with *Mycobacterium lepraemurium* (19). These suppressor cells, which are detectable around 2 months after an intravenous infection, belong, at least in part, to T lymphocytes displaying the $L3T4^+$ $Ly\text{-}2^-$ phenotype (19). After incubation in antigen- and mitogen-free culture medium, mycobacterial antigen-reactive cells spontaneously released suppressor factors in culture supernatants. Indeed, cell supernatants have the capacity to depress in vitro the proliferative response of normal spleen cells to mycobacterial antigens and to polyclonal mitogens. In addition, when adoptively transferred to syngeneic mice, they depressed the expression of the delayed-type hypersensitivity response to *M. lepraemurium* antigens, reduced the mean survival time of infected mice, and enhanced the growth of acid-fast bacilli at the site of infection and their dissemination to lymphoid and nonlymphoid organs (19). The observation that suppressor factor-containing culture supernatants can markedly depress the resistance of mice to *M. lepraemurium* infection prompted us to define the exact nature of these mediators.

The present results show that the depression of the proliferative response to specific antigens and polyclonal mitogens is caused by the presence of distinct molecules with suppressor activity in spleen cell culture supernatants of *M. lepraemurium*-infected mice. One of these suppressor mediators was identified with gamma interferon (IFN-γ). Tumor necrosis factor alpha (TNF-α) and TNF-β, at doses that do not affect blastogenic responses, were found to potentiate the suppressor activity of IFN-γ. Finally, the presence of cell-free T-cell receptor αβ (TCR-αβ) or its degradation products in culture supernatants can interfere with the blastogenic response to mycobacterial antigens.

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MATERIALS AND METHODS

Infection of mice. Female mice of the inbred strain C57BL/6 (Charles River Breeding Labs Inc., St.-Constant, Quebec, Canada) were maintained under standard laboratory conditions and fed Purina Chow and water ad libitum. They were infected intravenously with 10^7 living bacilli of the Hawaiian strain of *M. lepraemurium*. Nine weeks after infection, that is, when suppressor cells were detected in vitro (18), spleens were removed under sterile conditions and the lymphoid cells were isolated as previously described (24).

Isolation of mycobacterial antigen-reactive cells. Spleen cells were first separated into surface immunoglobulin-positive and -negative cells by a panning technique (28) in which spleen cells were layered on plastic petri dishes (100 mm) coated with affinity-purified goat anti-mouse immunoglobulin G (IgG) and IgM (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). The surface immunoglobulin-negative cells are hereafter referred to as the T cell-enriched population. The mycobacterial antigen-reactive cells were then isolated on petri dishes coated with mycobacterial antigens as described elsewhere (19). Briefly, 10 ml of a sonic extract of *Mycobacterium avium* (75 μg/ml) was poured into 100-mm-diameter dishes (Fisher Scientific Co.). The dishes were incubated for 24 h at 4°C and washed four times with 10 ml of Hanks balanced salt solution. The T cell-enriched population (5 ml; 10^7 cells/ml) was layered on the dishes and incubated for 1 h at 37°C in 5% CO_2. The nonadherent cells were removed by washing the dishes gently four times with 10 ml of warm (37°C) RPMI 1640 culture medium. The adherent (antigen-reactive) cells were harvested by flushing the dishes three times with 10 ml of chilled (4°C) culture medium.

Production of suppressor factors. The mycobacterial antigen-reactive cells at a density of 5×10^5 cells per ml of RPMI 1640 medium in the absence of antibiotics, fetal bovine

TABLE 1. Suppressive activity of culture supernatants of mycobacterial antigen-reactive spleen cells

Addition to cultures of normal spleen cells[a]	[^{3}H]thymidine incorporation (cpm [10^3]) induced with:			
	Antigens[b]	LPS	ConA	Control
RPMI 1640	14.9 ± 2.7[c]	20.2 ± 2.0	123.3 ± 6.5	1.0 ± 0.02
Supernatant from normal mice	15.8 ± 2.4	24.0 ± 1.8	111.3 ± 4.5	1.1 ± 0.01
Supernatant from infected mice	6.8 ± 0.6[d] (56.9%)	12.4 ± 2.3[d] (48.0%)	81.9 ± 10.6[d] (26.4%)	0.7 ± 0.01

[a] Normal spleen cells (3.5×10^5 cells per well of a 96-well culture plate) suspended in RPMI 1640 alone or in RPMI 1640 containing 50% (vol/vol) of culture supernatants obtained from mycobacterial antigen-reactive cells of normal or *M. lepraemurium*-infected mice were stimulated with antigen and mitogens as described in Materials and Methods. Thymidine incorporation was determined 72 h after cell stimulation.

[b] Mycobacterial antigens.

[c] Data represent the means of eight experiments ± standard errors of the means.

[d] Statistically different ($P < 0.05$ at least, as measured with the Student *t* test) compared with values obtained with spleen cell supernatants from normal mice. Values in parentheses represent the percent suppression, calculated from data obtained with spleen cell supernatants of normal mice.

serum, and mitogens or antigens were incubated for 12 to 15 h at 37°C in a humidified atmosphere of 5% CO_2. Supernatants were harvested by centrifugation (500 × *g*, 10 min), filtered through a 0.22-μm-pore-size membrane (Acrodisc no. 4192; Gelman Sciences Inc.), and stored at −20°C until use.

Assessment of suppressive activity. The suppressive activity of spleen cell culture supernatants (diluted 50% [vol/vol] in RPMI 1640) was evaluated by its capacity to inhibit the lymphoblastic transformation of normal spleen cells (3.5×10^6/ml) stimulated with concanavalin A (ConA; 5.0 μg/ml), lipopolysaccharide (LPS; 50 μg/ml), and mycobacterial antigens (150 μg/ml) as previously described (25). The suppressive activity was expressed as the percentage of suppression according to the equation $[1 - (a - c)/(b - c)] \times 100$, where *a* and *b* represent the mean counts per minute of the mitogenic response obtained with the spleen cell culture supernatant from infected and normal mice, respectively, and *c* represents the mean background counts per minute. In some experiments, various amounts of neutralizing units of a monoclonal anti-murine IFN-γ antibody (Lee Biomolecular Research Inc.) were added to culture supernatants for 1 h at room temperature before the cultures were tested for suppressive activity. To adsorb soluble TCR from culture supernatants, petri dishes (60 × 15 mm, no. 3002; Becton Dickinson) were coated with 10 ml containing 1.25 μg of the monoclonal antibody F23.1, which binds to the β chain (Vβ8) of the murine TCR (21), or 1.25 μg of mouse IgG (Miles Laboratories Inc.). Then 2.0 ml of culture supernatant was added, and the dishes were incubated at 25°C for 1 h. After two subsequent adsorptions under the same conditions, no soluble TCR, as determined with Western immunoblotting (see below), was detected in the culture supernatants. The suppressive property of recombinant mouse IFN-γ, recombinant mouse TNF-α, and recombinant human TNF-β (all from Genzyme Corp., Boston, Mass.) on the blastogenic response were also determined.

IFN-γ and TNF-α assays. The IFN-γ and TNF-α activities in spleen cell culture supernatants of normal and *M. lepraemurium*-infected mice were evaluated by using the mouse IFN-γ and the mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kits (Genzyme) according to the manufacturer's instructions. Since the presence of TNF-β can also be detected with the TNF-α ELISA kit, according to the manufacturer's instructions, the type of TNF detected by this kit will not be specified throughout this paper.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on culture supernatants under reducing conditions in 11% polyacrylamide slab gels as described by Laemmli (11). The gels were loaded with 30 μl of each sample (at a concentration of 500 μg of protein per ml). The electrophoresis was run overnight at a current of 6.0 mA, and the gels were stained with the Bio-Rad silver staining kit (Bio-Rad Laboratories).

Western immunoblotting. Samples (1 μl) of the culture supernatants (500 μg of protein per ml) were laid on nitrocellulose papers. The papers were saturated for 1 h with 3.0% bovine serum albumin in 0.05 M Tris buffer (pH 7.5) containing 0.15 M NaCl. Then, 150 neutralizing units (NU) of a rabbit anti-human TNF-β polyclonal antibody (Genzyme) per ml, 150 NU of a rabbit anti-human TNF-α polyclonal antibody (Endogen Inc., Boston, Mass.) per ml, and a 1/300 dilution (0.3 μg/ml) of the monoclonal anti-TCR antibody (F23.1) were added to the papers. After overnight incubation, the papers were washed three times with Tris buffer and incubated for 1 h with 0.5 μCi of iodinated protein A (Amersham Searle Corp., Oakville, Ontario, Canada) diluted in Tris buffer supplemented with 3.0% bovine serum albumin. The papers were then washed three times in Tris buffer containing 0.5% (vol/vol) Triton X-100 (Bio-Rad Laboratories), dried, laid on Kodak XAR-5 film with a screen magnifier, and stored at −90°C for 4 days. The film was developed with Kodak GBX developer.

Determination of protein content. The protein concentration of supernatants was determined by the Bio-Rad protein test kit and expressed as micrograms of protein per milliliter.

RESULTS

Suppressor activity of culture supernatants. Mycobacterial antigen-reactive cells isolated from the spleens of *M. lepraemurium*-infected and normal mice were incubated in serum- and mitogen-free culture medium for about 15 h at 37°C, and the culture supernatants were tested for the ability to depress the blastogenic responses of normal spleen cells to mycobacterial antigens, LPS, and ConA. In agreement with our previous results (18), the spleen cell culture supernatants from infected mice had the capacity to depress the blastogenic responses to all three activating agents, whereas the supernatants from normal mice were devoid of such activity (Table 1). Table 1 also shows that the suppressor activity (expressed as the percentage of suppression) was higher in cultures activated with specific antigens (57%) and LPS (48%) than in those activated with ConA (26%). Since mycobacterial extracts act mainly as a B-cell mitogen (22), these results suggested that the suppressor mediators present in culture supernatants would act preferentially on B lymphocytes rather than on T lymphocytes.

Electrophoresis of culture supernatants on SDS-PAGE. Aliquots of lyophilized culture supernatants containing 15 μg

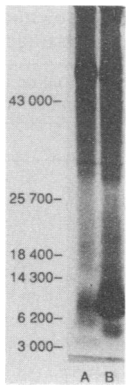

FIG. 1. SDS-PAGE analysis of spleen cell culture supernatants from normal and *M. lepraemurium*-infected mice. Lyophylized preparations (15 μg) of culture supernatants from normal (lane A) and infected (lane B) mice were electrophoresed on a 11% polyacrylamide gel and stained with silver. Molecular weight standards (indicated on the left): ovalbumin, 43,000; α-chymotrypsinogen, 25,700; β-lactoglobulin, 18,400; lysozyme, 14,300; bovine trypsin inhibitor, 6,200; insulin, 3,000.

of proteins were subjected to SDS-PAGE. Compared with culture supernatants from normal spleen cells, those from *M. lepraemurium*-infected mice contained at least three additional components of 27, 19.7, and 5.2 kDa and a larger amount of a 10.5-kDa product (Fig. 1). These additional components might represent, at least in part, cytokines produced by the mycobacterial antigen-reactive cells during the 15-h incubation, since these cells were likely activated at the time of their positive selection in petri dishes coated with specific antigens. Thus, the following experiments were designed to identify cytokines with suppressive activity in spleen cell culture supernatants of infected mice.

Detection of IFN-γ in spleen cell culture supernatants. In some studies, IFN-γ has been found to exert an inhibitory effect on B-cell proliferation (for a review, see reference 6). Our analyses of spleen cell culture supernatants revealed that supernatants derived from 5×10^5 mycobacterial antigen-reactive T cells per ml contained 72.8 ± 1.4 U (mean value from four experiments ± the standard error of the mean) of IFN-γ per ml, whereas supernatants from the same number of antigen-nonreactive T cells isolated from the spleens of *M. lepraemurium*-infected and normal mice contained 3.1 ± 0.09 and <0.45 U/ml, respectively.

In the next series of experiments, increasing doses of murine recombinant IFN-γ were added to cultures of normal splenocytes in an attempt to inhibit blastogenic responses to mycobacterial antigens, LPS, and ConA. IFN-γ depressed the blastogenic response to the three activating agents in a dose-dependent manner (Table 2). Interestingly, whatever the doses used, the responses to mycobacterial antigens and to LPS were depressed to a larger extent than was the response to ConA. Treatment of normal splenocytes with 50,000 U of IFN-γ did not depress their proliferative responses any further (data not shown). It should be noted that the depressive activity of 500 U of IFN-γ, which in fact represents 6 to 7 times the amount detected in spleen cell culture supernatants of infected mice (see above), was much lower than that of the whole culture supernatants, thus implying that other suppressor factors were present in culture supernatants. The suppressive activity exerted by 5,000 U of IFN-γ was almost completely neutralized by the addition of 100 NU of a monoclonal anti-IFN-γ antibody (Table 2).

TABLE 2. Suppressive effect of recombinant IFN-γ on the lymphoblastic transformation of normal spleen cells

Addition to spleen cells[a]	[^{3}H]thymidine incorporation (cpm [10^3]) by normal spleen cells stimulated with[b]:		
	Antigens[c]	LPS	ConA
None	18.5	39.2	105.4
IFN-γ (50 U)	16.3 (11.5)	33.6 (14.1)	105.2 (0.2)
IFN-γ (500 U)	15.5 (16.3)	29.9 (23.7)	95.7 (9.2)
IFN-γ (5,000 U)	10.0 (45.7)	19.3 (50.6)	84.6 (19.7)
IFN-γ (5,000 U) + anti-IFN-γ (100 NU)	17.2 (7.0)	36.3 (7.4)	100.8 (4.4)

[a] Recombinant IFN-γ at the indicated concentrations per ml and the anti-IFN-γ monoclonal antibody (100 NU/ml) were added to normal spleen cells 1 h before the onset of activation.
[b] Normal spleen cells (3.5×10^5 cells per well) were activated with optimal concentrations of mitogens and specific antigens (Materials and Methods). The data represent the arithmetic mean of a representative experiment done in triplicate. The values within parentheses are the percent suppression.
[c] Mycobacterial antigens.

Effects of anti-IFN-γ on the suppressive activity of culture supernatants. Spleen cell culture supernatants from infected and normal mice as controls were treated for 1 h at room temperature with increasing amounts of a monoclonal anti-IFN-γ antibody and then tested for suppressive activity. Although treatment with 1 NU of the antibody did not affect to a significant extent the suppressive activity of spleen cell culture supernatants of infected mice, treatment with 10 NU led to a marked reduction of the suppressive activity in cultures activated with all three activating agents (Table 3).

TABLE 3. Monoclonal antibody to IFN-γ partially reverses the suppressive activity of spleen cell culture supernatants from *M. lepraemurium*-infected mice

Origin of culture supernatant[a]	Treatment	[^{3}H]thymidine incorporation (cpm [10^3]) by normal spleen cells activated with[b]:		
		Antigens[c]	LPS	ConA
Normal	None	11.4	19.3	111.6
Infected	None	6.5 (42.7)	12.2 (36.7)	92.5 (17.1)
Normal	Anti-IFN-γ (1 NU)	10.5	17.8	115.9
Infected	Anti-IFN-γ (1 NU)	5.8 (44.5)	11.2 (36.9)	102.9 (11.3)
Normal	Anti-IFN-γ (10 NU)	11.3	19.5	111.4
Infected	Anti-IFN-γ (10 NU)	8.7 (22.7)	14.3 (26.7)	107.4 (3.6)
Normal	Anti-IFN-γ (100 NU)	11.4	19.9	109.5
Infected	Anti-IFN-γ (100 NU)	8.4 (26.2)	14.1 (29.4)	105.8 (3.3)
Normal	IgG1	11.4	19.6	110.0
Infected	IgG1	6.8 (39.6)	11.8 (39.7)	92.9 (15.5)

[a] Spleen cell culture supernatants from normal and *M. lepraemurium*-infected mice were treated with the indicated amounts of monoclonal antibody to IFN-γ or with 0.45 μg of a rat IgG1 (corresponding to 100 NU of anti-IFN-γ) and then tested for suppressive activity.
[b] Normal spleen cells were cultured at 3.5×10^5 cells per well of a microplate with polyclonal mitogens and mycobacterial antigens in the presence of 50% (vol/vol) treated or untreated spleen cell culture supernatants. The data represent the means of one representative experiment done in triplicate. The percent suppression is shown within parentheses.
[c] Mycobacterial antigens.

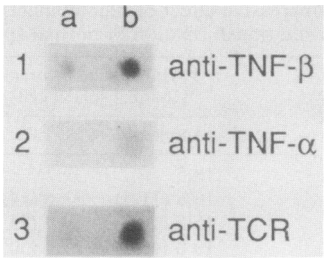

FIG. 2. Western immunoblotting (spot test) of culture supernatants from normal and *M. lepraemurium*-infected mice. Samples of 1 μl (500 μg of protein per ml) of culture supernatants obtained from antigen-reactive spleen cells from normal (a) and *M. lepraemurium*-infected mice (b) were laid on nitrocellulose papers. After incubation with a polyclonal anti-TNF-β antibody, a monoclonal anti-TNF-α antibody, and the monoclonal anti-TCR antibody (F23.1), the papers were treated with 0.5 μCi of iodinated protein A. For more details see Materials and Methods.

Treatment with 100 NU did not reduce any further the suppressive activity in culture supernatants. Finally, treatment with a rat IgG1 with the same isotype as the monoclonal antibody had no effect on the suppressive activity.

Involvement of TNF-α and TNF-β in the depressed blastogenesis. The above results suggested that suppressor factors other than IFN-γ were involved in the depression of the responses to mycobacterial antigens and LPS. A good candidate was TNF, because culture supernatants derived from mycobacterial antigen-reactive cells were found to be highly cytotoxic in vitro against either normal cells or transformed and tumorous cell lines (unpublished observations). Supernatants derived from 5×10^5 mycobacterial antigen-reactive cells per ml were found to contain 7.6 ± 3.1 U of TNF per ml (mean value from two experiments ± the standard error of the mean; each experiment was done in duplicate), whereas those from the same number of antigen-nonreactive cells isolated from the spleens of infected mice contained 2.18 ± 0.22 U/ml (data not shown). Less than 1.5 U of TNF per ml was detected in supernatants of antigen-nonreactive cells from normal mice. Since most of the mycobacterial antigen-reactive cells were T lymphocytes (19), we also looked for TNF-β. Western blot analysis revealed TNF-β in supernatants of antigen-reactive cells from infected mice, whereas those from normal mice contained much lower amounts of this lymphokine (Fig. 2, lane 1). TNF-β was more readily detectable than TNF-α (Fig. 2; compare lanes 1 and 2).

In other experiments, various doses of recombinant TNF-α and TNF-β were added to normal spleen cell cultures in an attempt to depress the blastogenic response to mycobacterial antigens and to mitogens. The antigen- and the LPS-induced blastogenic responses were not affected with either type of TNF used at doses as high as 5,000 U/ml; however, the response to ConA was significantly depressed at TNF-α and TNF-β doses of 1,000 U/ml and higher (data not shown). On the other hand, low doses of both types of TNF, when used in association with IFN-γ, can depress, in a dose-dependent manner, the proliferative responses to the three activating agents (Table 4). Indeed, the extent of the depression was higher in cultures treated with the mixture of lymphokines than in cultures treated with IFN-γ alone. This situation was interpreted as indicating that TNF-α and TNF-β acted by potentiating the suppressive activity of IFN-γ.

Involvement of soluble TCR-αβ in the depressed blastogenesis. It has been demonstrated that T-lymphocyte-derived suppressor factors were analogous to TCR-αβ (5, 16). By means of a Western blot analysis, the presence of TCR-αβ was readily demonstrated in culture supernatants of antigen-reactive cells from infected mice, whereas minute amounts of these receptors were detected in the corresponding supernatants obtained from normal mice (Fig. 2, lane 3).

Culture supernatants from infected mice were incubated in petri dishes coated with the anti-Vβ8 antibody F23.1 in an attempt to eliminate the soluble TCR-αβ molecules contained therein. After this treatment, these receptors were not detectable with Western immunoblotting analysis (data not shown). In addition, the suppressive activity of the TCR-depleted culture supernatants was markedly reduced in cultures activated with mycobacterial antigens but not in those activated with LPS and ConA (Table 5). Table 5 also

TABLE 4. TNF-α and TNF-β potentiate the suppressive activity of IFN-γ on the blastogenic response

Addition to spleen cells[a]	[^{3}H]thymidine incorporation (cpm [10^3]) by normal spleen cells stimulated with[b]:		
	Antigens[c]	LPS	ConA
None	17.7	36.4	155.0
IFN-γ (500 U)	15.3 (13.1)	32.6 (10.5)	134.2 (13.4)
IFN-γ (500 U) + TNF-α (10 U)	13.9 (23.7)	30.9 (15.1)	138.2 (11.0)
IFN-γ (500 U) + TNF-α (100 U)	12.3 (31.3)	30.3 (16.8)	133.8 (13.6)
IFN-γ (500 U) + TNF-β (100 U)	13.0 (25.5)	29.2 (19.8)	137.1 (11.3)
IFN-γ (500 U) + TNF-β (500 U)	12.4 (30.5)	25.8 (29.2)	124.7 (19.6)

[a] Recombinant TNF-α and TNF-β at the indicated doses in association with 500 U of recombinant mouse IFN-γ were added to normal spleen cells 1 h before the onset of activation.
[b] See footnote *b* of Table 2.
[c] Mycobacterial antigens.

TABLE 5. Reduction of suppressor activity after the removal of soluble TCR molecules from spleen cell culture supernatants of *M. lepraemurium*-infected mice

Origin of culture supernatants[a]	Treatment of supernatants	[^{3}H]thymidine incorporation (cpm [10^3]) by normal spleen cells activated with[b]:		
		Antigens[c]	LPS	ConA
Normal	Mouse IgG	11.4	27.8	130.6
	Anti-TCR	10.9	30.8	123.6
Infected	Mouse IgG	3.1 (72)	9.9 (64)	119.8 (9)
	Anti-TCR	5.5 (50)	9.6 (69)	121.4 (2)
	Anti-TCR + anti-IFN-γ	7.9 (28)	15.1 (51)	161.7 (0)
	Anti-IFN-γ	5.1 (53)	15.2 (51)	164.4 (0)

[a] Spleen cell culture supernatants from normal and *M. lepraemurium*-infected mice were layered on petri dishes previously coated with either murine IgG (control) or the anti-TCR monoclonal antibody (F23.1) as described in Materials and Methods. Where indicated, nondepleted and TCR-depleted supernatants were treated with 20 NU of anti-IFN-γ 1 h before they were tested for suppressive activity.
[b] Normal spleen cells were cultured at 3.5×10^5 cells per well of a microplate with mycobacterial antigens and polyclonal mitogens in the presence of 50% (vol/vol) treated or untreated cell culture supernatants. The data represent the means of one representative experiment done in triplicate. The percent suppression is shown within parentheses.
[c] Mycobacterial antigens.

shows that treatment of TCR-depleted supernatants with anti-IFN-γ antibodies further restored the response to mycobacterial antigens. In contrast, in LPS- and ConA-activated cultures the removal of free TCR molecules did not improve the level of restoration due to the anti-IFN treatment. Finally, since the combined treatment did not completely eliminate suppressor activity, it can be concluded that other suppressor mediators, acting alone or in synergy with those described in the present study, are still present in spleen cell culture supernatants of infected mice.

DISCUSSION

Our previous work (18) had shown that mycobacterial antigen-reactive cells, upon incubation in mitogen- or antigen-free culture medium, secrete nonspecific suppressor factors that depress the in vitro proliferative response of murine splenocytes to specific antigens and polyclonal mitogens. In the present experiments, we have confirmed this observation and extended it by showing that the depressed lymphoproliferation depends, at least in part, on the presence in culture supernatants of distinct suppressor mediators, namely, IFN-γ, TNF (acting as a potentiator of IFN-γ), and soluble TCR-α/β. The presence of IFN-γ and TNF (especially TNF-β) in culture supernatants is not surprising when one considers that mycobacterial antigen-reactive cells are positively selected on immobilized antigens; this process can provide the priming signal for cell activation and thus lead to lymphokine production. Mycobacterial antigen-reactive cells have been found to be rich in lymphocytes displaying the $L3T4^+$ $Ly\text{-}2^-$ phenotype (19). It is still unknown whether these cells are interleukin-2 (IL-2)-producing cells. However, since they can produce IFN-γ and TNF-β (see above), they might belong to the Th1 cells according to the classification proposed by Mosmann et al. (15).

The present experiments have shown the following: (i) relatively high levels of IFN-γ (ca. 70 U/ml) were present in culture supernatants of mycobacterial antigen-reactive cells; (ii) in agreement with the results of previous workers (8, 14), exogenous recombinant IFN-γ has the ability to depress the T- and B-cell proliferative responses in vitro (Table 2); and (iii) the suppressive activity of endogenous and exogenous IFN-γ can be neutralized by an anti-IFN-γ monoclonal antibody (Table 3). Together, these results identified IFN-γ as one of the nonspecific suppressive mediators present in spleen cell culture supernatants of *M. lepraemurium*-infected mice.

IFN-γ plays a major role in the activation of macrophages as demonstrated by the enhancement of their tumoricidal and microbicidal activities and the enhanced expression of major histocompatibility complex antigens, including the Ia antigen (for a review, see reference 3). In addition, activated murine splenic or peritoneal macrophages, unlike resident macrophages, can suppress the proliferative responses induced by polyclonal mitogens (23, 27). Thus, it is likely that the depression of proliferative responses, as observed in the present study, resulted from the activation by IFN-γ-containing supernatants (and also by the exogenous IFN) of the macrophages present in normal splenocytes, which were used for evaluating the suppressive activity. Further studies on the effects of IFN-γ on macrophage-depleted spleen cell cultures might confirm this interpretation.

IFN-γ may have opposite effects depending on the phenotype of T lymphocytes on which it is acting. For instance, in mixed lymphocyte cultures, IFN-γ can induce the expression of IL-2 receptors on $CD8^+$ T cells but not on $CD4^+$ T cells, thus enhancing the proliferation of $CD8^+$ cytotoxic T cells (20). On the other hand, Gajewski and Fitch (8) have reported that IFN-γ can inhibit the proliferation of Th2 cell lines. Since these cells produce IL-4, a B-cell growth factor, it has been postulated that the mechanism by which IFN-γ downregulates the proliferation of B lymphocytes might depend on a lack of IL-4 production (8). It is still unknown whether a lack of IL-4 production is involved in the IFN-γ-induced depression of the proliferative response to LPS, as observed in the present study. Interestingly and for still unknown reasons, the proliferative response of B lymphocytes was depressed to a larger extent by IFN-γ than was that of T lymphocytes. This observation suggests that the depressed proliferation of T and B lymphocytes would depend on two different mechanisms.

TNF-α and TNF-β, when used at doses devoid of cytotoxic activity, were able to potentiate the suppressive effect of IFN-γ (Table 4). A synergistic anti-proliferative and anti-tumoral property of TNF and IFN-γ has been reported by several other groups of workers (12, 17, 26). Similarly, Esparza et al. (4) found that TNF-α and TNF-β, either alone or in association with IFN-γ, activated macrophages and thus increased their oxidative respiratory burst and their tumoricidal and schistosomicidal activities. Since IFN-γ can enhance the expression of the TNF-α and TNF-β receptors on macrophages (1), these observations strongly suggest that TNF-α and TNF-β can act in synergy with IFN-γ for the activation of macrophages and, as discussed above, that activated macrophages exert suppressor activity.

The presence of soluble TCR-β chains was demonstrated in culture supernatants of mycobacterial antigen-reactive suppressor cells, and these receptors inhibited the response of normal splenocytes to mycobacterial antigens. The close relationship between suppressor factors and the β chain of the TCR has recently been established (5, 16). Our preliminary work, designed to further characterize the physicochemical nature of the suppressive TCR-β, revealed three low-molecular-mass fractions (28, 14.5, and 7.5 kDa) with the ability to inhibit the response of normal spleen cells to mycobacterial antigens. In addition, these fractions maintained their ability to react with the monoclonal anti-TCR-β F23.1 antibody. Therefore, it is likely that soluble degradation products of the β chain in culture supernatants can act as specific suppressor mediators. These mediators might interfere with the recognition process of the mycobacterial antigens by the T cells. Nevertheless, these results strongly suggest that the TCR-β chain can be released in part or as a whole from suppressor cells. It is still unknown whether the shedding of TCR-β from T lymphocytes represents a suppressor mechanism itself or a consequence of one.

Additional experiments will be needed to determine the specificity, the mode of action, and the physical nature of the TCR-β chain as well as its relationship with other suppressor mediators. Furthermore, the possibility that culture supernatants from antigen-reactive suppressor cells contain other suppressive lymphokines, such as IL-3 (13), IL-4 (7), TGF-β (9, 10), and soluble immune response suppressor (2), etc., cannot be excluded. So far, the involvement of prostaglandin E_2 as a suppressive entity in the supernatant of *M. lepraemurium*-infected mice has been eliminated (data not shown).

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