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Production of a Suppressor Factor by CD8$^+$ Lymphocytes Activated by Mycobacterial Components

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The lipid component present in high-molecular-mass fractions with molecular masses of >200 kDa derived from *Mycobacterium tuberculosis* extracts passaged through Sephacryl S.200 columns activate CD8$^+$ lymphocytes to suppress lymphocyte blastogenesis. Suppression is mediated by the release of suppressor molecules by these CD8$^+$ lymphocytes. Release of suppressor molecules occurs as early as 2 h following pulsing with the high-molecular-mass mycobacterial components and is maximal at 24 h, after which their release declines rapidly. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting indicates that the active components are carbohydrate moieties with approximate molecular masses of 122 to 148 kDa. Our results suggest a mechanism of interaction between mycobacteria and host mononuclear cells such that mycobacterial lipids, once exposed, activate CD8$^+$ suppressor lymphocytes. Activation of these lymphocytes results in the release of carbohydrate-containing molecules that ultimately inhibit the blastogenesis of other lymphocytes.

Immunosuppressive mechanisms operative in active tuberculosis have been well documented (5, 16, 17). These include immune suppression directly mediated by mycobacterial products (9) or mycobacterial manipulation of the host's immune suppressive mechanisms (26, 29). The latter mechanism results in suppression of host immunity due to monocytes or activation of adherent and nonadherent suppressor cells (14, 24, 26, 29). Such immune suppression has been demonstrated to be specific to mycobacteria (2, 17) or generalized because of an overwhelming mycobacterial load, as evidenced in tuberculous meningitis (2, 12, 16). Regardless of the mechanisms operative, immune suppression is a common feature during most mycobacterial infections (7).

We have previously demonstrated that when cultured human peripheral blood adherent cells ingest mycobacteria, they release suppressor cell-activating factors which induce CD8$^+$ cells to inhibit lymphocyte proliferation and lymphokine production (26, 27, 29). Subsequent studies demonstrated that these suppressor cell-activating factor molecules were lipids of mycobacterial origin (28).

The present study was undertaken to examine the effects of factors derived from *Mycobacterium tuberculosis* on CD8$^+$ cell functions. A further aspect of our study investigated the mechanisms of action of these suppressor cells in suppressing immune function. Our studies indicate that upon activating CD8$^+$ cells in vitro, these cells release a second soluble factor, which results in the suppression of lymphocyte proliferation.

MATERIALS AND METHODS

Mononuclear cell (MN cell) preparation. MN cells from the peripheral blood of normal, healthy volunteers were isolated by sedimentation on Hypaque-Ficoll gradients and washed and suspended in RPMI 1640 (MA Bioproducts, Walkersville, Md.) containing 2 mM L-glutamine and 50 μg of both penicillin and streptomycin per ml, and supplemented with 10% fetal calf serum (C-RPMI). Adherent cells were separated from nonadherent lymphocytes (NALs) by incubation on plastic petri dishes at 37°C for 1 h as previously described (6).

Enriched lymphocyte populations were obtained by incubating 5 μl of reconstituted monoclonal anti-CD4 or anti-CD8 antibodies (Orthoclone; Ortho Pharmaceutical Corp., Raritan, N.J.) per 10^6 cells at room temperature for 45 min. Fresh rabbit complement was then added to the cell suspensions, followed by a further incubation at 37°C for 1 h. Cell death, to determine the efficacy of such treatment, was determined by trypan blue exclusion. Viable cells remaining after antibody and complement treatment were recovered by centrifugation for 20 min at 4°C on Hypaque-Ficoll gradients. Enrichment of CD4$^+$ and CD8$^+$ lymphocytes was confirmed by further incubation with antibody and complement and was always >90%.

Preparation of mycobacterial fractions. *M. tuberculosis* organisms were obtained by scraping colonies off Lowenstein-Jensen slopes. Organisms were heat killed and homogenized, and mycobacterial extracts were obtained by sonication in an MSE Soniprep 150 ultrasonic disintegrator (MSE Scientific Instruments, Sussex, United Kingdom). The sonicated material was fractionated on Sephacryl S-200 columns as previously described (25). Fractions separated on these columns were arbitrarily pooled according to major protein peaks and labelled A through J. Of the 10 peaks so defined, fractions A through E were present in the void volume (25). Delipidation of *M. tuberculosis* extracts was achieved by treatment with 2 volumes of anesthetic ether. The mixture was frozen at −70°C and thawed at 37°C, and the interface containing any lipid removed. This was repeated three times, until the lipid was no longer observed at the interface. Lipid samples were pooled, and ether was removed by evaporation over nitrogen. The remaining lipid was reconstituted in 1 ml of C-RPMI containing 0.1 ml of ethanol and sonicated in an MSE ultrasonic disintegrator. Reconstituted materials were then brought to the original volume in C-RPMI. The remaining delipidated fractions

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were also reconstituted to the original starting volume in C-RPMI.

Lymphocyte proliferation assay. MN cells or adherent cell-depleted lymphocytes were cultured in round-bottom microtiter plates containing 2×10^5 cells/well. Cells were stimulated with the mitogen phytohemagglutinin (PHA; Wellcome Diagnostics, Dartford, United Kingdom) or concanavalin A (Con A; Pharmacia, Upsalla, Sweden) at a concentration of 10 μg/ml or with purified protein derivative (PPD; Connaught Laboratories, Toronto, Canada) at a concentration of 25 μg/ml. All experiments were performed in triplicate. Plates were incubated at 37°C in 5% CO_2 in humidified air for 72 h. Proliferation studies that employed PPD as the antigen source were undertaken with cells from known PPD-positive donors. Cultures were incubated for 5 days with 25 μg of PPD per ml. Tritiated thymidine ([*methyl*-^{3}H]thymidine; specific activity, 24 Ci/mmol) (Amersham, Buckinghamshire, England) at 1 μCi was added for the final 18 h of culture, after which the cells were harvested onto glass fiber filters by using an automated cell harvester 550 (Flow Laboratories, Irvine, Calif.). Radioactivity was counted by liquid scintillation with an LKB 1217 RackBeta liquid scintillation spectrometer (Bromma, Sweden). Lymphocyte proliferation is expressed as the mean ± the standard deviation of counts per minute of radioactivity incorporated by triplicate cultures of 2×10^5 cells. To assess the effects of mycobacterial fractions on lymphocyte proliferation, these fractions were added directly to cultures at a final concentration of 10 μg/ml.

Preparation of suppressor molecules from NAL. NALs at 2×10^6/ml were pulsed for 2 h at 37°C with unfractionated and various fractions of *M. tuberculosis* extracts that were obtained by sizing on Sephacryl S-200 columns at a concentration of 10 μg/ml (protein concentration as determined by the Bio-Rad protein assay kit) (Bio-Rad Laboratories, Richmond, Calif.). In addition, NALs were incubated with the lipid and delipidated components of these fractions. At the end of this pulsing period, cells were washed three times, suspended in C-RPMI to their original concentration, and incubated in tissue culture tubes for various periods of time. Cell-free supernatants were collected at 2, 4, 24, and 48 h and at 3, 4, and 5 days and stored at −20°C until used. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot analysis, 24-h supernatants were prepared by incubating pulsed NALs in phosphate-buffered saline (PBS).

Chemical modification of lymphocyte suppressor molecules. Carbohydrate moieties were degraded according to the method of Owhashi et al. (20). NAL-derived suppressor molecules (supernatants from 2×10^6 NALs/ml) were incubated in 0.05 M sodium metaperiodate (Merck, Darmstadt, Germany) in the dark with gentle mixing for 72 h at 4°C. The reaction was terminated by dialysis against distilled water (3 dialyses of 2 liters each at 4°C) and PBS (3 dialyses of 2 liters each at 4°C) for 48 h. Degradation of proteins was performed by incubating the NAL-derived suppressor molecules with 1 μg of proteinase K (Boehringer Mannheim, Germany) per ml for 1 h at room temperature. Delipidation of NAL-derived suppressor molecules was achieved by treatment with anesthetic ether as described for mycobacterial extracts. Lipid was reconstituted in 1 ml of RPMI 1640 containing 0.1 ml of ethanol and sonicated in an MSE ultrasonic disintegrator and then brought to the original volume in C-RMPI. The remaining delipidated fractions were also reconstituted to the original starting volume in C-RPMI.

Sialic acid residues were removed or destroyed by incubating the NAL-derived suppressor molecules with 50 U of neuraminidase (Boehringer Mannheim) per ml for 1 h at room temperature, followed by extensive dialysis (25).

SDS-PAGE and Western blot immunoassay. SDS-PAGE analysis of NAL-derived suppressor molecules was performed by loading 150 μg of 10× concentrated supernatants onto gels of 10% acrylamide under nonreducing conditions (11). Samples were electrophoresed in a Tris–glycine–SDS buffer (0.2 M Tris-HCl, 1.92 M glycine, 1% SDS) system by using a Bio-Rad Protean II slab gel apparatus under conditions of constant current (25 mA per gel). Protein markers were identified on gels by silver staining (Quick Silver silver staining kit; Amersham), whereas glycoprotein-containing bands were stained with Schiff's reagent (Clinical Sciences Diagnostics, Booysens, Johannesburg South Africa) (31).

Electroblotting of unstained gels was performed under conditions of constant current and voltage (0.3 A; 70 V) for 3 h (23). The Bio-Rad Transblot cell apparatus (Bio-Rad Laboratories) was used for electroblotting.

Solubilized suppressor molecules from Western blots were prepared according to published protocols (1). After completion of Western blotting, the nitrocellulose sheet was rinsed and both control lanes and suppressor molecule lanes were cut into 2-mm^2 pieces, sterilized with dimethyl sulfoxide, and used in proliferation assays.

RESULTS

Suppression of lymphocyte blastogenesis mediated by mycobacterial fractions. When eluates of mycobacterial extracts present in the void volume of Sephacryl S-200 columns were added to nonadherent cultures stimulated with PHA and Con A, a significant reduction in [^{3}H]thymidine uptake was observed (Table 1). These fractions were composed of proteins, carbohydrates, and lipids. Suppression was evident with as little as 2 μg/ml (final concentration) of mycobacterial extract and reached a plateau at concentrations greater than 10 μg/ml (results not shown). Unfractionated mycobacterial extracts or fractions eluting with molecular masses below 200 kDa were without effect.

In order to ascertain the component(s) in these high-molecular-mass fractions that was inducing suppression, experiments were undertaken whereby the fractions were pooled and delipidated, and the lipid and delipidated fractions were added to cultures stimulated with mitogens. Suppression of lymphocyte blastogenesis was lost only when delipidated fractions were employed (Table 1). When the lipid components from these fractions were reintroduced into cultures, suppression of lymphocyte blastogenesis was again observed. Similar effects could be observed in assays employing lymphocytes incubated with PPD (Table 1). Fractions with molecular masses of >200 kDa and the lipid components from these fractions were capable of suppressing the proliferative responses of nonadherent lymphocytes to this antigen.

The effects of supernatants from NALs pulsed with various mycobacterial components. Our previous studies indicated that lipid components of mycobacteria activated function rather than increased the numbers of T suppressor cells (29). Experiments were therefore undertaken in which cell-free supernatants were collected at various intervals after pulsing NALs with high-molecular-mass fractions or the lipid and delipidated components of these fractions. Control supernatants were derived from cultures pulsed with whole unfractionated mycobacterial extracts. The results indicate that supernatants from NALs incubated with high-molecular-

TABLE 1. Effects of mycobacterial fractions on lymphocyte blastogenesis

Culture systems	Mean cpm ± SD of 5 experiments (% suppression)[a]
Nonadherent lymphocytes alone with:	
Medium	1,277 ± 223
PHA	51,180 ± 4,667
Con A	51,285 ± 4,135
PPD	17,048 ± 437
Nonadherent lymphocytes incubated with:	
Unfractionated mycobacterial extracts plus:	
Medium	1,273 ± 321
PHA	50,981 ± 3,762 (0)
Con A	54,105 ± 3,102 (0)
PPD	17,134 ± 2,140 (0)
Fractions of <200 kDa plus:	
Medium	2,306 ± 882
PHA	49,802 ± 3,160 (3)
Con A	50,612 ± 2,917 (1)
PPD	16,827 ± 1,070 (1)
Fractions of >200 kDa plus:	
Medium	1,973 ± 832
PHA	33,621 ± 2,972 (34)
Con A	35,367 ± 3,981 (31)
PPD	9,214 ± 1,495 (46)
Delipidated fractions of >200 kDa plus:	
Medium	2,895 ± 735
PHA	48,460 ± 3,746 (5)
Con A	48,547 ± 4,135 (5)
PPD	17,296 ± 1,895 (0)
Lipid components from fractions of >200 kDa plus:	
Medium	1,224 ± 772
PHA	35,850 ± 3,398 (30)
Con A	32,180 ± 3,721 (37)
PPD	10,107 ± 1,207 (41)

[a] Suppression is calculated by comparison with cpm of [^{3}H]thymidine of the respective lymphocyte cultures stimulated with PHA, Con A, or PPD.

mass fractions, or fractions containing lipids, were capable of suppressing the blastogenesis of fresh untreated lymphocytes stimulated with PHA (Fig. 1). Significant suppressor activity was evident in these supernatants as early as 2 h following pulsing and maximal suppression of lymphocyte proliferation was observed when 24-h supernatants were used in such assays. This suppression gradually declined and was no longer seen after 72 h. Suppression was not due to a cytotoxic effect of the supernatants on lymphocytes as evidenced by trypan blue exclusion tests at the termination of experiments (results not shown). Supernatants derived from NALs incubated with mycobacterial fractions of molecular masses of >200 kDa were therefore termed suppressor supernatants, whereas those from NALs incubated with low-molecular-mass fractions were referred to as control supernatants. Supernatants from control cultures pulsed with unfractionated mycobacterial extracts and those pulsed with delipidated high-molecular-mass fractions did not induce such suppression (Fig. 1).

Identification of lymphocytes responsible for secreting suppressor molecules. To identify the lymphocyte subset responsible for releasing suppressor molecules, experiments were undertaken whereby NALs and enriched $CD4^+$ and $CD8^+$ lymphocyte populations were pulsed for 2 h with fractions with molecular masses of >200 kDa or <200 kDa. After washing, 24-h supernatants from the respective cultures were incubated with fresh lymphocytes stimulated with PHA. Supernatants from enriched $CD8^+$ lymphocytes, pulsed with high-molecular-mass mycobacterial fractions, significantly suppressed lymphocyte blastogenesis in response to PHA (Table 2). No such suppressor activity was observed in supernatants from $CD4^+$ lymphocytes or from untreated $CD8^+$ cells or from $CD8^+$ cells treated with mycobacterial fractions with molecular masses below 200 kDa.

Characterization of suppressor molecules in $CD8^+$ culture supernatants. To further characterize the components responsible for inducing suppression, 24-h supernatants from $CD8^+$ lymphocytes pulsed with high-molecular-mass mycobacterial fractions were subjected to treatments that removed lipid or degraded carbohydrate or polypeptide portions and sialic acid residues. Destruction of sialic acid residues had no effect on the suppressive ability of these supernatants. When supernatants containing only lipids and carbohydrates (in which the protein moieties were destroyed) were used, suppression of lymphocyte blastogenesis also remained unaffected (Table 3). Suppressor activity was, however, no longer evident in supernatants that were treated with sodium metaperiodate. Furthermore supernatants in which carbohydrate and protein moieties were destroyed (leaving mainly lipid components) no longer induced the suppression of lymphocyte blastogenesis (Table 3).

When supernatants that induced suppression of lymphocyte blastogenesis were resolved by SDS-PAGE, protein staining demonstrated major bands at ~67 and 150 kDa. Staining for carbohydrate moieties demonstrated bands in the region of ~122 to 148 kDa, ~90 kDa, ~55 kDa, and ~50 kDa (Fig. 2B, lane C). Control cultures or those that were not immunosuppressive contained bands in the regions of ~90 kDa, ~55 kDa, and ~50 kDa. These included supernatants from $CD4^+$ cultures incubated with high-molecular-mass mycobacterial fractions (Fig. 2B, lane A), supernatants from $CD8^+$ cultures incubated with mycobacterial fractions of molecular masses of <200 kDa (Fig. 2B, lane B), and supernatants from unstimulated NALs (Fig. 2B, lane D). Other control systems, such as mycobacterial extracts with molecular masses of <200 kDa (Fig. 2A, lane E) or of molecular masses of >200 kDa (Fig. 2A, lane F) or the medium control containing C-RPMI only (Fig. 2A, lane G), though staining for protein and carbohydrate, did not demonstrate bands in the same areas as the test systems (Fig. 2A and B).

Following transfer onto nitrocellulose membranes, areas corresponding to identifiable carbohydrate or protein bands were excised and used in proliferation assays. Suppression of lymphocyte blastogenesis in response to PHA was evident only with molecules present in the ~122- to 148-kDa region (Fig. 3). No suppressor activity was evident in regions above and below this range (Fig. 3). When corresponding nitrocellulose strips from control culture supernatants were employed, no such suppressor activity could be detected. Control systems that contained supernatants from $CD4^+$ cells pulsed with mycobacterial fractions with molecular masses of >200 kDa or $CD8^+$ cells pulsed with mycobacterial fractions with molecular masses of <200 kDa or mycobacterial extracts alone did not induce suppression of lymphocyte blastogenesis after Western blotting in these assays

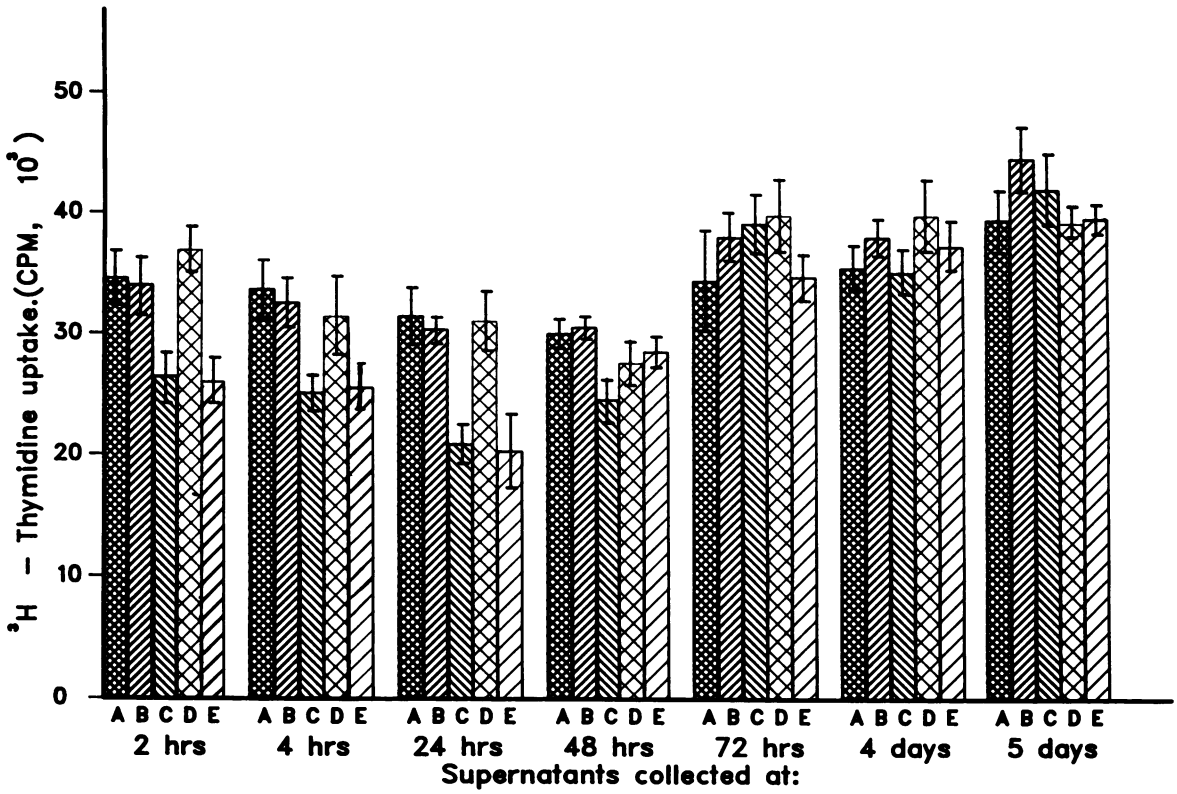

FIG. 1. Effects of various supernatants on lymphocyte blastogenesis in response to PHA (mean ± standard deviation of five experiments). Supernatants were collected from cultures pulsed with medium (A), unfractionated mycobacterial extracts (B), fractions of mycobacterial extracts in the void volume of Sephacryl S.200 columns (C), and delipidated (D) and lipid (E) components of these fractions and incubated with fresh lymphocytes in the presence of PHA. Significant suppression ($P < 0.005$) of lymphocyte blastogenesis was observed in the presence of mycobacterial extracts with molecular masses of >200 kDa (C) and fractions containing mycobacterial lipids (E) at 2 and 24 h.

(results not shown). Nitrocellulose strips corresponding to any of the other carbohydrate-staining bands were also without effect (Fig. 3). In addition, the two bands that stained for protein also did not induce suppression of lymphocyte blastogenesis (results not shown).

TABLE 2. Effects of supernatants from CD4+- and CD8+-enriched populations pulsed with mycobacterial fractions on lymphocyte blastogenesis

24-h supernatants from CD4+- or CD8+-enriched T-cell populations incubated with:	Blastogenic response of NALs (% suppression)[a]
Medium	
CD4+	49,653 ± 2,290
CD8+	50,329 ± 2,543
Fractions of <200 kDa	
CD4+	49,521 ± 2,311 (0)
CD8+	49,982 ± 2,943 (0)
Fractions of >200 kDa	
CD4+	48,503 ± 1,981 (2)
CD8+	37,470 ± 1,846 (26)

[a] Values are means of cpm ± standard deviation for three experiments. Uptake of [^{3}H]thymidine was in the presence of PHA. Percent suppression was calculated by comparison with cpm of respective CD4+ and CD8+ lymphocyte cultures pulsed with PHA and incubated in medium alone. The mean cpm ± standard deviation of NALs incubated with PHA alone in these experiments was 48,892 ± 2,183.

DISCUSSION

Previous studies from this laboratory have demonstrated that human peripheral blood adherent cells incubated with mycobacteria release mycobacterial lipids that activate suppressor cells (26–29). The present study undertook to extend these findings by examining the effects of various fractions of *M. tuberculosis* on nonadherent lymphocytes and, in particular, their interaction with suppressor lymphocytes.

The results of the present study demonstrate that unfractionated mycobacterial sonic extracts do not affect the blastogenic response of NALs to mitogenic stimulation. This is in keeping with our previous findings indicating that suppression due to mycobacteria was dependent on the presence of adherent macrophages (29) and suggesting that some processing by macrophages is required.

In an attempt to identify components present in mycobacterial sonic extracts that were responsible for such suppression, studies were undertaken whereby mycobacterial sonic extracts were separated on Sephacryl S-200 columns. Fractionation by size resulted in 10 major fractions, of which 5 appeared in the exclusion volume of such columns (25). Only these high-molecular-mass fractions (>200 kDa) suppressed lymphocyte blastogenesis (results not shown). Fractions were therefore pooled and divided into two groups: those with a molecular mass of >200 kDa and those with a lower molecular mass. Such an artificial division indicated that pooled fractions with molecular masses of >200 kDa consistently suppressed the proliferative responses of nonadherent

TABLE 3. Effects of chemically modified suppressor supernatants on lymphocyte blastogenesis

Culture systems	Mean cpm ± SD of 3 experiments (% suppression)[a]	
	PHA	PPD
NALs incubated with:		
Medium	41,326 ± 1,642 (0)	17,048 ± 437 (0)
Control supernatant	49,521 ± 2,311 (0)	15,101 ± 1,456 (11)
Suppressor supernatant	21,530 ± 1,556 (47.9)	8,843 ± 669 (41)
Suppressor supernatant chemically modified with:		
Neuraminidase	24,882 ± 1,431 (40)	9,501 ± 1,364 (44)
Proteinase K	21,179 ± 1,263 (49)	8,216 ± 1,509 (52)
Sodium metaperiodate	41,746 ± 1,378 (0)	17,693 ± 1,329 (0)
Proteinase K plus sodium metaperiodate	38,433 ± 1,991 (7)	16,195 ± 798 (5)

[a] Percent suppression was calculated by comparison with the counts per minute (cpm) of [^{3}H]thymidine of the respective lymphocyte cultures stimulated with PHA and PPD and incubated in medium alone. SD, standard deviation.

lymphocytes (Table 1). Because our previous studies indicated that activation of suppressor function was mediated by the lipid components of *M. tuberculosis* (26, 28, 29), we removed and isolated the lipid in the high-molecular-mass fractions. Our results indicate and confirm that mycobacterial lipids are responsible for suppressing T lymphocyte proliferation (Table 1). Our results also suggest that in vitro fractionation of mycobacterial antigens results in exposure of mycobacterial components that do not require processing by macrophages for T-cell recognition. Of these fractions, only the high-molecular-mass components result in suppression of lymphocyte blastogenesis.

High-molecular-mass mycobacterial antigens have been implicated in suppression of immune responses in other studies (2, 19). Even though the exact nature of T-cell unresponsiveness in lepromatous leprosy remains controversial, evidence favors the role of $CD8^+$ suppressor T lymphocytes in mediating the specific anergy seen in leprosy (2). Further indirect evidence favors the role of suppressor cells by suggesting that mycobacterial antigens with molecular masses of >150 kDa appear to selectively activate T lymphocytes from anergic patients with lepromatous leprosy and not those from tuberculoid leprosy (19). Suppression due to such high-molecular-mass bacterial products is not unique to mycobacteria, as evidenced by a report in which a 185-kDa streptococcal cell wall antigen has been shown to activate $CD8^+$ lymphocytes (15).

In order to examine the mechanism by which suppression

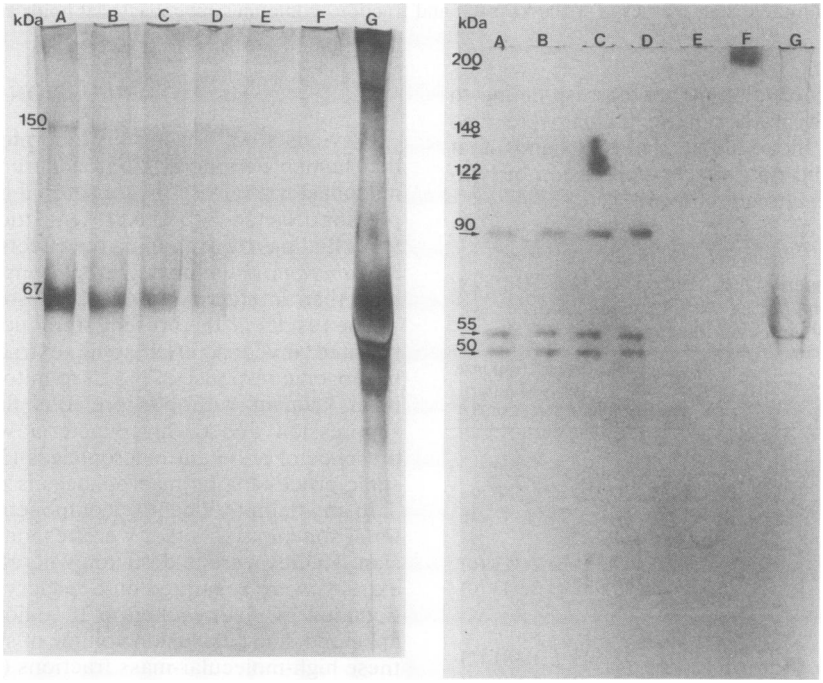

FIG. 2. SDS-PAGE of 24-h supernatants from $CD4^+$ lymphocytes, $CD8^+$ lymphocytes, and NALs. (A) Supernatants from $CD4^+$ cultures incubated with mycobacterial fractions with molecular masses of >200 kDa; (B) Supernatants from $CD8^+$ cultures incubated with mycobacterial fractions with molecular masses of <200 kDa; (C) Supernatants from $CD8^+$ cultures incubated with mycobacterial fractions with molecular masses of >200 kDa; (D) Supernatants from unstimulated NAL cultures; (E) *M. tuberculosis* extracts with molecular masses of <200 kDa; (F) *M. tuberculosis* extracts with molecular masses of >200 kDa; (G) C-RPMI (medium control). The arrows indicate the approximate molecular masses for protein-staining bands (silver stain) (left panel) and carbohydrate-staining bands (Schiff's) (right panel).

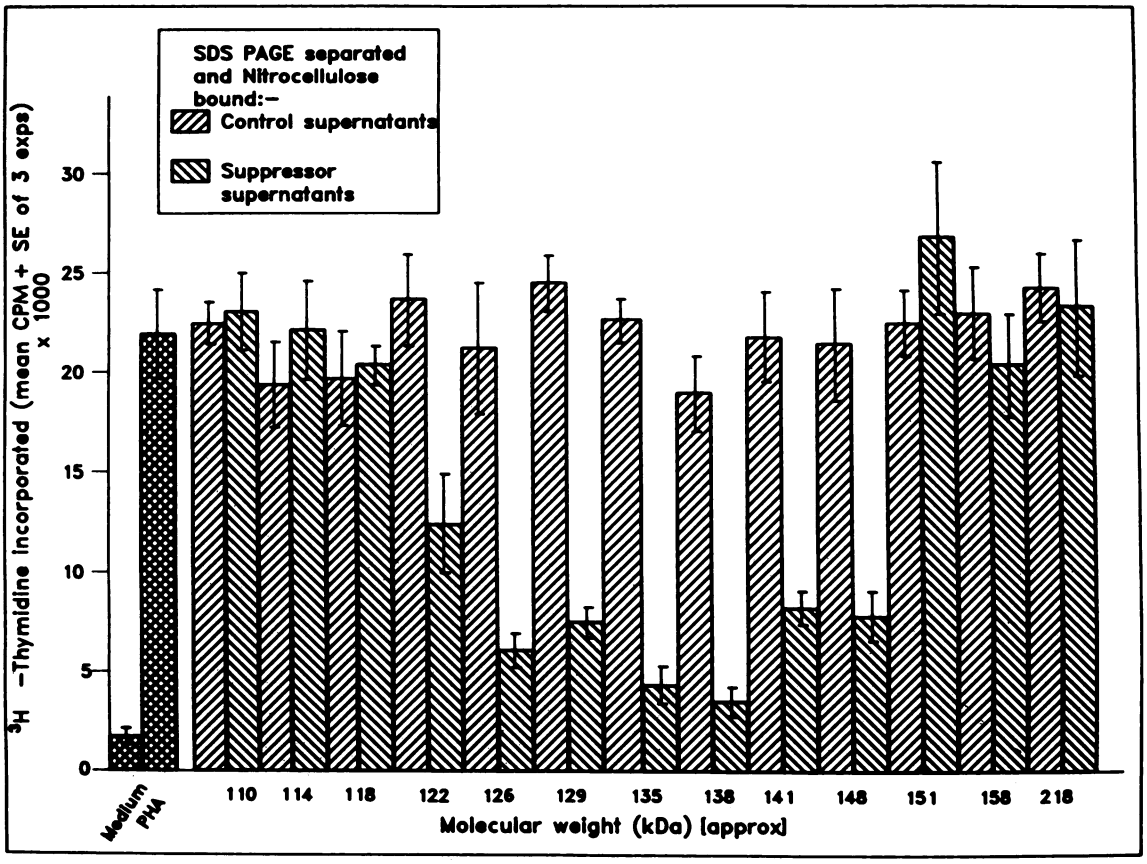

FIG. 3. Lymphocyte proliferation in response to nitrocellulose-bound supernatants that had been separated by SDS-PAGE. Significant suppression of lymphocyte blastogenesis was observed only when fractions with molecular masses of from 122 to 148 kDa from suppressor supernatants were employed ($P < 0.001$).

of lymphocyte blastogenesis occurred, experiments utilizing cell-free supernatants from NALs pulsed with high-molecular-mass fractions were incubated with fresh NALs in the presence of PHA. Suppression of lymphocyte blastogenesis appeared to be mediated by the release of a second group of suppressor molecules, as evidenced by the fact that NALs pulsed with the high-molecular-mass components (or lipids from these components) and washed extensively thereafter release soluble suppressor molecules which suppressed lymphocyte blastogenesis. Release of these "second messengers" was evident within 2 h of culture, and was maximal at 24 h. Since the culture system employed utilized pulsed, washed cells, followed by washing after each subsequent incubation period, our results favor suppression resulting from the release of such a second messenger rather than from contaminating mycobacterial components. This is further borne out by our preliminary identification of the molecules by SDS-PAGE (Fig. 2) as being different from the mycobacterial lipids previously described (26–29). It is also of interest that unfractionated mycobacterial extracts do not induce the release of such second messengers (Fig. 1, columns B), nor do they suppress lymphocyte blastogenesis (Table 1). Release of such suppressor molecules from lymphocytes could not be induced even after 5 days in culture with unfractionated mycobacterial sonic extracts. This was also true of NALs pulsed with delipidated high-molecular-mass fractions. The latter confirms the previously described activation of suppressor cell function by lipids of mycobacterial origin (26, 28). Furthermore, suppression of lymphocyte blastogenesis was not due to the release of cytotoxic factors by activated lymphocytes (assessed by trypan blue exclusion), nor were these supernatants effective on resting NALs (results not shown).

To identify the cell type that is activated in mediating suppression, 24-h supernatants from enriched populations of $CD4^+$ or $CD8^+$ lymphocytes pulsed with high-molecular-mass mycobacterial fractions were incubated with fresh NALs in the presence of PHA or PPD. Our results indicate that only supernatants from enriched $CD8^+$ cultures were capable of suppressing lymphocyte blastogenesis (Table 2), confirming previous reports that such suppression is due to the activation of $CD8^+$ lymphocytes (2, 19, 26, 28). Preliminary characterization of the second messenger indicates that the active components in $CD8^+$ supernatants are carbohydrate moieties (Table 3). This is further confirmed by SDS-PAGE, which demonstrates the staining of carbohydrate and little staining of protein. Experiments with nitrocellulose membranes onto which these molecules were transferred indicate suppressor activity in the molecular mass region of approximately 122 to 148 kDa, which corresponded to Schiff-staining areas in this region (Fig. 2B). Even though proteins were identified by SDS-PAGE, these were in the molecular mass regions of ~67 and 150 kDa (Fig. 2A) and did not affect lymphocyte proliferation, nor did carbohydrate-containing bands with molecular masses of ~90, 55, and 50 kDa, detected in supernatants of control

cultures (results not shown). In order to exclude the possibility that failure of suppression by these proteins or carbohydrates was due to lack of transfer, the nitrocellulose strips were stained for proteins following Western blotting. Our results, not presented here, confirmed successful transfer of these molecules. Furthermore, isolated nitrocellulose strips covering the entire area of transferred control supernatants separated by SDS-PAGE were not immunosuppressive (Fig. 3).

Release of soluble suppressor molecules has been described in several systems. These include their release by mononuclear cells activated by Con A (10), by UV-induced T suppressor cells (30), and by splenic T cells from mice injected with a high dose of *M. bovis* BCG (4). Attempts at characterizing such suppressor molecules have identified them as soluble saccharides with molecular masses of 30 to 40 kDa (10), 45 to 60 kDa (30), 50 to 70 kDa (3), 60 to 80 kDa, and 100 to 200 kDa (22), and other reports suggest the presence of polysaccharides of *Candida albicans* (8) and an 85-kDa uromodulin (18) in serum to be immunosuppressive. The results of the present study demonstrating the release of suppressor molecules with approximate molecular masses of 122 to 148 kDa by activated $CD8^+$ cells is therefore by no means novel but suggests that such mechanisms may also be operative in mycobacterial infections.

Of the various components of mycobacteria, arabinomannan (6), lipoarabinomannan (13), phenolic glycolipid-1 (21), and D-arabino-D-galactan (7) have been demonstrated to be immunosuppressive. The present study, however, suggests that, once stimulated by mycobacterial lipids, suppressor cells release carbohydrate-like moieties that are immunosuppressive. Our results suggest that the carbohydrate-mediated suppression we describe does not act via interference with mitogens, since lymphocyte blastogenesis in response to PPD is similarly suppressed. Even though we have not as yet identified the exact carbohydrate responsible for the observed immune suppression, results from other studies in this laboratory suggest that such suppression is mediated by interference with cytokine production.

Our findings, together with our previous reports (29), suggest that mycobacterial lipids activate $CD8^+$ suppressor lymphocytes to release carbohydrate-containing molecules with approximate molecular masses of 122 to 148 kDa which suppress lymphocyte blastogenesis.

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