Suppression of Lymphocyte Responses by Tuberculous Plasma and Mycobacterial Arabinogalactan

MONOCYTE DEPENDENCE AND INDOMETHACIN REVERSIBILITY

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ABSTRACT During tuberculosis, exposure of monocytes to circulating factors may induce the suppressor activity observed in some anergic patients. To explore this possibility, we examined the effects of plasma pooled from 28 untreated tuberculosis (TB) patients and the mycobacterial cell wall polysaccharide D-arabino-D-galactan (AG) on the in vitro function of peripheral blood mononuclear cells (PBMC) from healthy donors. In the [^{3}H]thymidine incorporation assay, stimulated responses of PBMC incubated in culture medium supplemented with TB plasma or co-cultured with 3.0 μg/ml AG were depressed significantly when compared with control responses. Cytotoxicity and altered kinetics of stimulated DNA synthesis did not contribute to the observed suppression. TB plasma and AG-induced suppression of the PBMC response to purified protein derivative was monocyte dependent and indomethacin reversible. In addition, TB plasma and AG directly inhibited the phytohemagglutinin-stimulated responses of T lymphocytes. In a quantitative assay of monocyte attachment to plastic, both TB plasma and AG significantly increased monocyte adherence from basal levels. These effects on monocyte adherence were reversed with indomethacin or antibody to mycobacterial polysaccharide. In addition, TB plasma passed over an immunoabsorbent column of Sepharose-linked antibody to mycobacterial polysaccharide was depleted of the suppressive and monocyte-adherence augmenting factors. 3.0 μg/ml AG stimulated a fivefold increase in prostaglandin E_2 production by cultured mononuclear cells. Our data suggest that AG circulating alone or bound in immune complexes may account for the observed effects of TB plasma. Similar in vivo exposure may contribute to the cell-mediated suppression of lymphocyte responses in tuberculosis.

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INTRODUCTION

Several observations suggest that suppressor cells contribute to the impaired cellular immunity characteristic of chronic infection. Circulating adherent cells that suppress T lymphocyte responses to the infecting agent occur in schistosomiasis (1, 2), filariasis (3), leprosy (4), and tuberculosis (5). Neither the basis for antigen specificity nor the mode of action of these adherent monocyte-enriched suppressor cells is known. In tuberculosis, monocytes also suppress the B cell response to pokeweed mitogen in hemolytic plaque-forming cell assays (6). In chronic fungal infection, suppressor-adherent cells mediate a nonspecific inhibitory effect (7). These suppressor cells produce a soluble factor that stabilizes an otherwise labile suppressor T lymphocyte population (8). Suppressor T lymphocytes with surface receptors for the Fc portion of IgG develop in tuberculosis (9) and leprosy (4), but their relationship to the suppressor monocytes remains undefined. Prostaglandin-secreting suppressor monocytes have been described in healthy individuals (10); increased production of immunosuppressive prostaglandin by monocytes may underlie the nonspecific suppression in Hodgkin's disease (11). However, in tuberculosis, the antigen-specific suppression by monocytes is not reversible with indomethacin (5).

Presumably in infectious diseases, suppressor cells result from exposure to the parasite or factors derived from the host-parasite interaction. Parasite prod-

ucts that suppress in vitro lymphocyte responses to specific antigens have been described in chronic mucocutaneous candidiasis (12), schistosomiasis (1), leprosy (13), and tuberculosis (14). Moreover, autologous serum inhibits in vitro responses of lymphocytes from patients with schistosomiasis (15), histoplasmosis (16), filariasis (3), and tuberculosis (17). Our study of tuberculosis explored whether suppressive plasma factors, possibly mycobacterial in origin, alter monocyte function, and thereby induce immunosuppression during the course of infection. We examined the effect of tuberculosis patient plasma and the mycobacterial polysaccharide D-arabino-D-galactan (AG)[1] on in vitro function of mononuclear cells obtained from healthy donors.

METHODS

Subjects. Blood donors in these studies were healthy volunteers of known tuberculin reactivity. Except as noted, donors had a positive delayed skin test reaction to 5 tuberculin units of tuberculin-purified protein derivative (PPD).

Mitogens, antigens, and drugs. Phytohemagglutinin (PHA) M-form was purchased from Grand Island Biological Co., Grand Island, N. Y. PPD was the gift of Lederle Laboratories, Div. American Cyanamid Co., Wayne, N. J. Streptokinase-streptodornase (SKSD) was obtained commercially (Lederle Laboratories) and dialyzed to remove preservatives. The PHA concentration (1/500 dilution) was selected to produce submaximal lymphocyte stimulation in the [^{3}H]thymidine incorporation assay; responsiveness to submaximal mitogen stimulation discriminates between the lymphocyte function of healthy and diseased populations (18). The antigen concentrations (10 μg/ml PPD, 1/400 dilution SKSD) stimulated optimal lymphocyte responses. Indomethacin (Sigma Chemical Co., St. Louis, Mo.) was prepared at 10 mg/ml in 95% ethyl alcohol, then diluted in RPMI 1640 for cell culture. At the final indomethacin concentration of 1 μg/ml, no functional effect of the alcohol diluent was apparent.

Pooled tuberculosis patient (TB) plasma. Plasma was obtained from 28 patients with newly diagnosed, culture-proven, pulmonary TB. Aliquots of each plasma sample were pooled and stored at −20°C. Before use in the [^{3}H]thymidine incorporation assay, plasma was heated at 56°C for 30 min.

Mycobacterial polysaccharides, arabinomannan (AM), and AG. The mycobacterial polysaccharides were prepared from 8–10 wk cultures of *Mycobacterium tuberculosis* H37Ra (Trudeau Institute, Saranac Lake, N. Y.) grown on Proskauer-Beck medium. AM and AG were isolated from the supernate of 50% saturated ammonium sulfate precipitation of the culture filtrates by Concanavalin A affinity chromatography (18). Chemical and immunological analyses of AM and AG as prepared for use in these experiments have been published (19, 20). Before use in cell culture, lyophilized polysaccharides were reconstituted in 0.15 M phosphate-buffered saline (PBS), pH 7.2, filtered through a 0.2-μm membrane, and centrifuged at 100,000 *g* for 60 min. The polysaccharide concentration was measured by the orcinol method (21) of pentose determination and expressed as micrograms pentose per milliliter. At AG concentration ≤1.0 μg/ml, there was no endotoxin contamination by a limulus lysate assay sensitive to endotoxin at concentrations ≥ 10 ng/ml. Materials used in purification of the mycobacterial polysaccharides did not affect mitogen- or antigen-induced lymphocyte [^{3}H]thymidine incorporation (14).

Cell populations. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Hypaque sedimentation of heparinized (20 U/ml) blood (22). PBMC consisted of 55–75% neuraminidase-treated sheep E-rosetting cells, or T lymphocytes (23), 10–15% surface immunoglobulin-bearing cells or B lymphocytes (24), and 15–25% monocytes by peroxidase-staining characteristics (25). Adherent cell depletion was effected by culture on a plastic petri dish for 1 h at 37°C followed by incubation of the nonadherent cells on a 600-mg, acid-washed nylon wool column (24). This adherent cell-depleted, T lymphocyte-enriched population contained 70–95% sheep E-rosetting cells with <1% contamination by B cells or monocytes (5). Cells dislodged from plastic petri dishes by gentle scraping with a rubber policeman were >75% monocytes by peroxidase-staining characteristics.

[^{3}H]Thymidine incorporation assay. Responder cells were cultured in microtiter plate tissue culture wells (Falcon Labware, Div. Becton Dickinson, Oxnard, Calif.,) at 1.5×10^5/0.1 ml. The tissue culture medium RPMI 1640 was supplemented with 50 U/ml sodium penicillin-G, 5 μg/ml gentamicin, 2 mM L-glutamine, and included 10% by volume heat-inactivated pooled human serum (PHS) or TB plasma. Mitogen-induced [^{3}H]thymidine incorporation was assayed following a 3-d culture in flat-bottom microtiter wells incubated at 37°C in 5% CO_2. Antigen-induced responses were assayed after 5-d culture in round-bottom microtiter wells. 1 μCi [^{3}H]thymidine, specific activity 5.0 Ci/mmol (Radiochemical Centre, Amersham, England) was added to each well 18 h before termination of culture. The cells were harvested and washed on filter paper disks using a semi-automated microharvester (Microbiological Associates, Walkersville, Md.). Disks were placed in vials to which ACS scintillant (Radiochemical Centre) was added and ^{3}H activity was determined spectrophotometrically. Results are expressed as mean counts per minute of quadruplicate wells or Δcpm (antigen or mitogen-stimulated cpm minus unstimulated cpm).

The significance of results was evaluated with the Student's *t* test for unpaired samples or the *t* test for paired samples.

Assay of monocyte attachment to plastic. PBMC at 1×10^6 cells/0.5 ml RPMI 1640 supplemented 5% by volume with heat-inactivated fetal calf serum (FCS; KC Biological Inc., Lenexa, Kans.) were cultured in 16-mm plastic wells (Linbro Chemical Company, Hamden, Conn.) 1 h at 37°C. The plates were washed three times with Hanks' Balanced Salt Solution (HBSS; KC Biological Inc.) to remove the nonadherent cells. 30 mM xylocaine in HBSS was added to the adherent monolayer, and, after 20 min, the cells were dislodged with a rubber policeman and enumerated in a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.). This adherent cell population is 90–95% peroxidase positive (25). Monocyte adherence to plastic was expressed as the mean number of cells recovered from triplicate wells. The significance of results was analyzed using Student's *t* test.

Preparation of goat antibody to mycobacterial polysaccharide. An adult female goat was immunized with complete Freund's adjuvant containing *M. tuberculosis* H37Ra. Antibody was identified and characterized in test bleedings using immunoelectrophoresis against whole *M. tuberculosis*

[1] *Abbreviations used in this paper: AG, arabinogalactan;* AM, arabinomannan; FCS, fetal calf serum; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; PHA, phytohemagglutinin; PHS, pooled human serum; PNP, pooled normal plasma; PPD, purified protein derivative; SKSD, streptokinase-streptodornase; TB, tuberculosis.

culture filtrate (26). The serum used in these studies reacted only with *M. tuberculosis* antigens 1 and 2 (27), AM and AG, respectively. Before use in the monocyte attachment assay, the immunoglobulin fraction was separated from goat serum by precipitation in 33% saturated ammonium sulfate. This precipitate was dialyzed and reconstituted in PBS to a 5-mg protein/ml concentration.

Preparation of immunoabsorbent columns. Goat antibody to mycobacterial polysaccharide was reacted with cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). Affinity columns, each with a 5-ml bed volume, were prepared with this immunoabsorbent (28). TB plasma or pooled normal plasma (PNP) in 1.0-ml aliquots was applied to each column and eluted with PBS. The eluate was lyophilized and reconstituted to the original volume in PBS.

Determination of PBMC prostaglandin E_2 (PGE_2) production. The PGE_2 concentration in supernates of cultured PBMC was determined by Dr. Michael J. Dunn using a radioimmunoassay (29). PBMC at 5×10^6 cells/ml were cultured in RPMI 1640 supplemented with PHS, 10% by volume, or supplemented with PHS and 3.0 μg/ml AG. The cultures were incubated 18 h at 37°C in 5 CO%. After incubation, the cells were sedimented by centrifugation and the supernatant fractions harvested. The PGE_2 concentration of the supernatant fraction is expressed as nanograms per milliliter per 10^6 cells.

RESULTS

Inhibition of [^{3}H]thymidine incorporation by pooled TB plasma. The effect of TB plasma on the response of PBMC is shown in Fig. 1. In each experiment, cultures of PHA- or PPD-stimulated PBMC in TB plasma-supplemented RPMI 1640 significantly decreased the stimulated DNA synthesis when compared with the PHS-supplemented PBMC cultures ($P < 0.005$, paired t test).

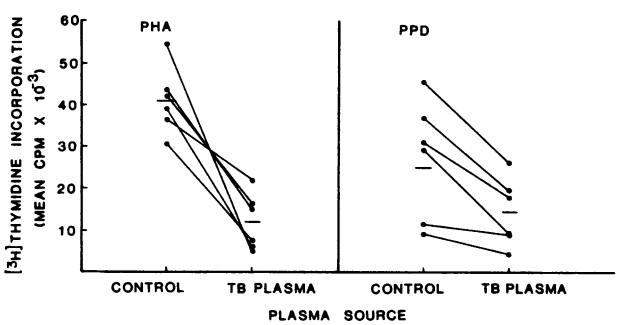

FIGURE 1 Suppression of the response of PBMC to PHA and PPD by TB plasma. The data are presented as mean counts per minute of quadruplicate microtiter wells. Lines connect the responses of PBMC of the same donor. Control responses in PHS-supplemented RPMI 1640 are compared with the response obtained in TB plasma-supplemented medium. The mean response (horizontal bar) to PHA was decreased from 41,446±3,303 (mean cpm±SEM) to 9,797 ±2,977 by TB plasma ($P < 0.001$, Student's t test). In PPD-stimulated cultures, the mean control response of 24,584 ±4,232 was reduced to 11,824±2,514 by TB plasma ($P < 0.05$, Student's t test).

We demonstrated comparable responses of stimulated PBMC when cultured in RPMI 1640 supplemented by PHS or PNP. Therefore, in the control experiments, the culture medium was supplemented with PHS because of its greater stability after heat inactivation and refrigeration.

To assess whether the inhibition of [^{3}H]thymidine incorporation by TB plasma represented suppression by circulating factors or resulted from inadequate support of the cell culture, TB plasma was diluted in PHS before addition to cell suspensions. In three experiments, undiluted TB plasma inhibited the PBMC response to PPD by a mean 41±5% ($P < 0.02$, Student's t test), whereas TB plasma diluted 50% with PHS suppressed PPD-stimulated responses by 46±9% ($P < 0.02$, Student's t test). At a 1/10 dilution in PHS, TB plasma no longer inhibited the PBMC response to PPD.

We also examined the effects of TB plasma on unstimulated PBMC and on the kinetics of stimulated DNA synthesis. TB plasma did not alter the [^{3}H]-thymidine incorporation of unstimulated PBMC after 3 or 5 d incubation in eight experiments. In cell cultures supplemented with PHS or TB plasma, DNA synthesis was assayed at 24-h intervals. In this experiment (Fig. 2), the peak responses of the TB plasma-supplemented cultures, although decreased in magnitude, coincided with the peak response of PHS-cultured PBMC. Another aspect of kinetics was studied by delaying the addition of TB plasma to stimulated cell cultures. TB plasma effected suppression only when added during the initial 24 h incubation.

To explore the specificity of inhibition by TB plasma, we studied its effect on the SKSD response. In six experiments, TB plasma inhibited the SKSD response by a mean 51±9% ($P < 0.002$, Student's t test). To examine whether susceptibility to suppression by TB plasma required prior mycobacterial infection, we

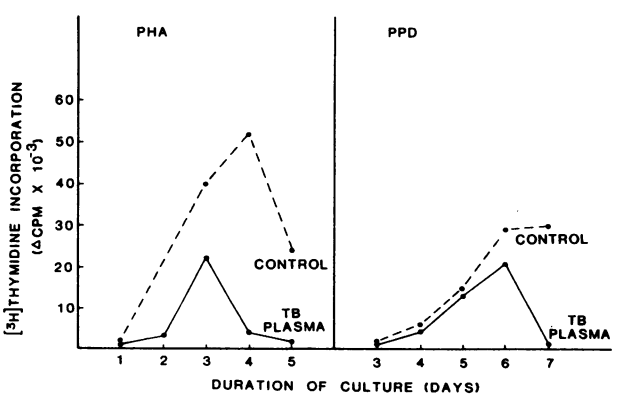

FIGURE 2 The time-course of suppression of PBMC cultures by TB plasma. The [^{3}H]thymidine incorporation of PBMC supplemented with PHS (----) or TB plasma (——) was assayed at 24-h intervals through the incubation period.

TABLE I

Effect of TB Plasma on [^{3}H]Thymidine Incorporation by T Lymphocytes Stimulated with PHA or PPD*

Experiment	Response to PHA		Response to PPD	
	PHS‡	TB plasma	PHS	TB plasma
	cpm±SD		*cpm±SD*	
1	38,989±5,369	19,546±1,666	149,826±15,937	126,880±23,494
2	5,523±725	1,978±168	10,363±1,017	11,418±3,191
3	8,721±1,667	2,257±1,337	62,016±16,313	54,383±2,331
4	37,454±4,180	11,333±1,618	31,089±3,498	34,109±5,690
5	38,395±5,902	8,670±3,900	32,385±3,217	32,502±1,616
6	9,098±1,691	737±21	14,961±2,165	13,663±1,089
7	8,207±1,136	2,043±192	30,368±1,705	14,987±3,081

* T lymphocytes were prepared by depletion of cells adherent to plastic and nylon wool (<1.0% monocytes by peroxidase cytochemistry).

‡ Tissue culture medium RPMI 1640 supplemented 10% by volume with PHS or TB plasma.

studied its effect on the PBMC responses of tuberculin-negative donors. In these three experiments, TB plasma suppressed the PHA response by a mean of 62±8% ($P < 0.02$, Student's t test).

The suppression of PBMC responses by TB plasma could result from direct inhibition of T lymphocytes or from effects of TB plasma mediated indirectly through other cell populations in the cultures. To distinguish between these possibilities, [^{3}H]thymidine incorporation was studied in T lymphocytes depleted of cells adherent to plastic and nylon wool. In each of seven experiments (Table I), T lymphocytes cultured in TB plasma were less responsive to PHA than cells from the same donor cultured in PHS-supplemented medium. However, in six of seven experiments, TB plasma did not suppress the T lymphocyte response to PPD. Cells dislodged from plastic petri dishes (>75% monocytes) were added to T lymphocytes to approximate the ratio of monocytes to T lymphocytes in unfractionated PBMC (0.3×10^5 monocytes + 1.2×10^5 T lymphocytes per microtiter well). In five experiments, TB plasma inhibited the responses to PPD of this reconstituted cell population by 38±11% ($P < 0.002$, Student's t test). This series of observations suggested that the circulating factor(s) in TB plasma modulate the antigen response through monocytes in the cell culture. TB plasma directly inhibited the T lymphocyte response to PHA. To examine whether the T lymphocytes or monocytes were altered by exposure to TB plasma, we added precultured cells to fresh responder cells of the same donor. The T lymphocytes and monocytes were incubated for 48 h in RPMI 1640 supplemented with TB plasma. After vigorous washing and counting, the T cells or monocytes were added to freshly prepared T lymphocytes of the same donor. Under the usual culture conditions for antigen or mitogen stimulation, these precultured cells did not inhibit the stimulated responses of T lymphocytes.

Suppression of [^{3}H]thymidine incorporation by AG. AM, a cytoplasmic polysaccharide, is an immunosuppressive mycobacterial product in the [^{3}H]thymidine incorporation assay (14). We compared its potency with that of AG, a chemically distinct, mycobacterial cell wall polysaccharide. In co-culture experiments, AM at a concentration of 40 μg/ml inhibited the antigen but not the PHA responses of PBMC. AG, over a broad concentration range (Fig. 3), consistently and significantly suppressed PHA and PPD-stimulated DNA synthesis. AG, therefore, is the more potent immunosuppressive mycobacterial polysaccharide;

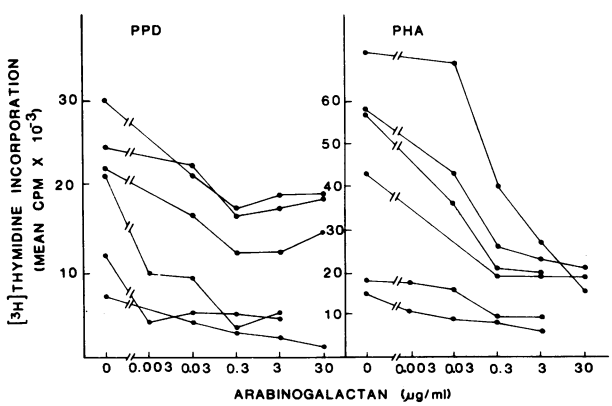

FIGURE 3 Suppression of PBMC responses by varied concentrations of AG in co-culture. The solid line connects [^{3}H]thymidine incorporation of PBMC elicited by a constant PHA or PPD stimulus as the concentration of AG in co-culture increased from 0.003 to 30 μg/ml. When these responses were compared to those of control cell suspensions, significant suppression occurred at all AG concentrations ≥0.03 μg/ml ($P < 0.02$, Student's t test).

subsequent studies of its immunoregulatory potential were performed at a 3.0 μg/ml concentration.

In each experiment with 3.0 μg/ml AG present in co-culture (Fig. 3), the PBMC response to PHA or PPD was decreased significantly when compared with control responses ($P < 0.005$, paired t test). AG in co-culture decreased the mean response to PHA from 49,370±7,447 to 20,591±2,746 ($P < 0.005$, Student's t test). The PPD response was inhibited by a mean 36±6% ($P < 0.002$, Student's t test).

To assess whether AG was intrinsically cytotoxic or mitogenic, we examined its effect in unstimulated PBMC cultures. In three experiments, PBMC cultured for 48 h in RPMI 1640 supplemented with PHS and 3.0 μg/ml AG had >75% survival and 95% viability by trypan blue dye exclusion. Survival and viability of AG-exposed PBMC did not differ from that of control, unexposed PBMC. In six experiments, AG had no effect on [^{3}H]thymidine incorporation of unstimulated PBMC after 3 or 5 d of cell culture.

The kinetics of AG suppression of PBMC responses were examined in time-course experiments and by varying the time of addition of AG to the stimulated cell cultures. AG did not accelerate or delay the PBMC response to PHA or PPD when compared with control responses, although the stimulated DNA synthesis was absolutely decreased by AG. In three experiments, the addition of AG to stimulated PBMC culture was delayed 24 h. AG still inhibited the PHA response by 69±29% ($P < 0.05$, Student's t test) and the PPD response by 25±9% ($P < 0.005$, Student's t test). Later additions of AG did not suppress the response of stimulated PBMC cultures.

Cell separation and recombination experiments were performed to ascertain the cell requirements for AG suppression of PBMC responses. Although PBMC response to PPD was decreased by AG in each of six experiments (Fig. 4, $P < 0.005$, paired t test), the T lymphocyte response was not altered consistently nor the mean response inhibited significantly by AG in co-culture. Reconstitution of the PBMC population by addition of plastic adherent cells (>75% monocytes) to T lymphocytes restored susceptibility to AG suppression. The PPD response of this reconstituted cell population was inhibited by 26±7% ($P < 0.002$, Student's t test) in six studies. In each experiment, AG decreased the PBMC and the T lymphocyte response to PHA ($P < 0.05$, paired t test).

T lymphocytes and monocytes precultured for 48 h in AG-supplemented medium did not suppress the antigen and mitogen responses of fresh T lymphocytes obtained from PBMC of the same donor.

Indomethacin reversal of TB plasma and AG suppression of PBMC responses. We next explored the reversibility of suppression of PBMC responses by 1.0 μg/ml indomethacin. At this concentration, indomethacin inhibits in vitro production of PGE_2 by monocytes (10). In these experiments (Fig. 5), indomethacin increased [^{3}H]thymidine incorporation of stimulated PBMC exposed to TB plasma or AG. Indomethacin did not affect the T lymphocyte response to PHA in either PHS or TB plasma-supplemented medium (Table II).

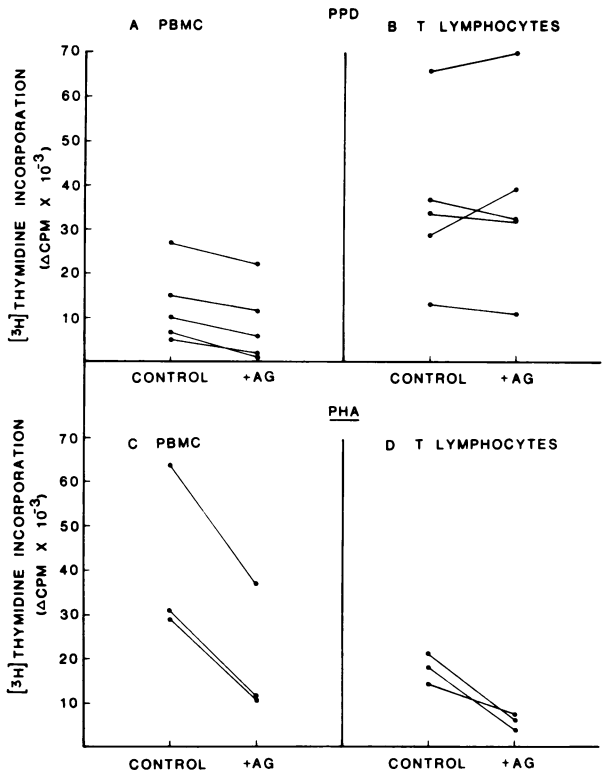

FIGURE 4 Comparison of the effect of AG on the PBMC and T lymphocyte responses to PPD and PHA. The data are presented as Δcpm (Δcpm = experimental cpm minus unstimulated cpm) with lines connecting the responses of cells from the same donor in cultures without or with AG. AG decreased the PBMC response to PPD (panel A) and to PHA (panel C). The PPD response of the T lymphocytes prepared by adherent cell depletion (panel B) was not suppressed by AG in co-culture. The mean PPD response of control T lymphocytes (35,561±11,223) was not different from T lymphocytes co-cultured with AG (38,383±12,328). However, the mean PHA response of control T lymphocytes of 18,003±1,933 was decreased to 6,044±770 ($P < 0.005$, Student's t test) by AG (panel D).

Alteration in monocyte adherence to plastic by TB plasma and AG. We studied whether TB plasma and AG altered the adherence properties of monocytes using a quantitative assay of monocyte attachment to plastic. After 1 h incubation in 16-mm plastic wells, both TB plasma and AG increased the monocyte adherence from the control level (Table III, $P < 0.002$). AG in concentrations from 0.03 to 3.0 μg/ml increased monocyte attachment ($P < 0.05$) with the maximal ef-

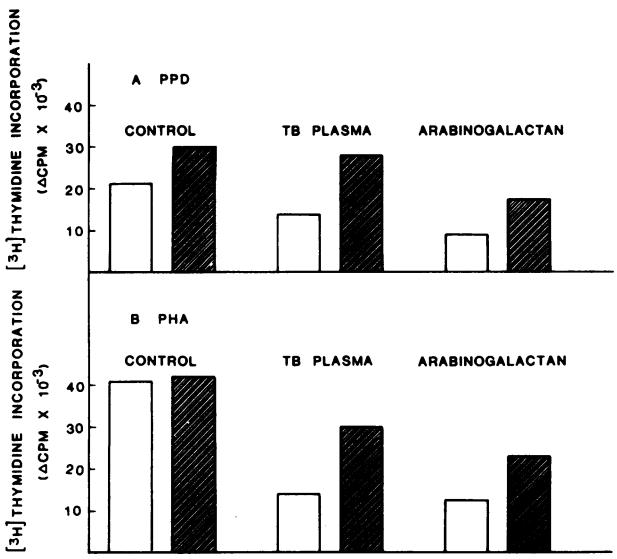

FIGURE 5 Effect of indomethacin on TB plasma and AG suppression of PBMC responses to PPD (panel A) and to PHA (panel B). Data are presented as the mean response in Δcpm for PBMC cultured without (open bars) or with (hatched bars) 1.0 μg/ml indomethacin. Data are shown for control cell suspensions and for cells cultured with TB plasma or AG. (A) PPD stimulation. The mean response of control PBMC (21,538±4,373) was not significantly different from the mean response with indomethacin, 33,344±6,063 in nine experiments. The mean response in TB plasma, however, was increased from 14,270±2,819 to 27,794±5,648 by the addition of indomethacin ($P < 0.05$, paired t test) whereas the mean response of AG co-cultured PBMC increased from 9,947±4,676 to 20,540±5,701 ($P < 0.05$, paired t test) with indomethacin. The mean responses of the indomethacin-treated cells did not differ from the control response. (B) PHA stimulation. The mean response of control PBMC was not affected by the addition of indomethacin. The mean response in TB plasma increased from 14,205±4,877 to 30,233±3,993 ($P < 0.05$, paired t test) in the presence of indomethacin. The mean response of these TB plasma-exposed, indomethacin-treated PBMC was not different from the control response. Despite an increase in the response of AG co-cultured cells from 12,581±2,239 to 23,289±5,631 with indomethacin ($P < 0.05$, paired t test), the mean response of AG co-cultured, indomethacin-treated cells was less than the control response ($P < 0.05$, Student's t test).

fect at 0.03 μg/ml. The TB plasma and AG augmentation of monocyte adherence was decreased significantly ($P < 0.02$) when 1.0 μg/ml indomethacin was added to PBMC suspensions.

AG stimulates in vitro PGE_2 production. Indomethacin reversibility of the effect of AG on stimulated DNA synthesis and monocyte adherence to plastic suggested that increased monocyte prostaglandin production mediated the effect of this mycobacterial polysaccharide. To assess whether AG stimulated PGE_2 production, PBMC were cultured in control medium or with 3.0 μg/ml AG. In each of five experiments

TABLE II
Effect of Indomethacin on T Lymphocyte Response to PHA*

Experiment	PHS‡		TB plasma‡	
	Indomethacin§		Indomethacin	
	Δcpm		Δcpm	
1	35,917	38,602	7,804	7,793
2	35,762	43,487	10,819	12,758
3	8,259	8,453	1,643	2,196

* T lymphocytes were prepared by depletion of cells adherent to plastic and nylon wool.
‡ RPMI 1640 supplemented 10% by volume with PHS or TB plasma.
§ 1 μg/ml indomethacin added to cell suspensions.

(Table IV), AG stimulated PGE_2 production. The mean basal production of immunoreactive PGE_2 7±2 ng/ml/10^6 cells was increased to 39±10 ng/ml/10^6 cells ($P < 0.02$) by co-culture with AG.

Antimycobacterial polysaccharide antibody prevents increments in monocyte adherence induced by TB plasma and AG. The TB plasma used throughout these studies contained IgG antibody to mycobacterial AG at a titer of 1/640 as determined with an enzyme-linked immunoassay (30). IgM antibody to AG was present at plasma dilution of 1/160. Using the orcinol method of pentose determination sensitive to 2 μg/ml, no free AG could be detected in this plasma pool.

Goat antibody to the mycobacterial polysaccharides and the immunoglobulin fraction of normal goat serum were mitogenic for PBMC in the [^{3}H]thymidine incorporation assay but did not alter base-line monocyte adherence in the assay of monocyte attachment to plastic. The specific antibody to mycobacterial poly-

TABLE III
Effect of TB Plasma, AG, and Indomethacin on Monocyte Attachment to Plastic

Addition to PBMC	Cells attached per well±SEM (×10^{-3})
Nil*	54±1.0
TB plasma‡	67±2.0
TB plasma + 1.0 μg/ml indomethacin	55±1.0
AG, 0.03 μg/ml§	68±1.0
AG + 1.0 μg/ml indomethacin	57±2.0

* Control wells contained 1 × 10^6 PBMC in RPMI 1640+5% by volume FCS. Substitution of PHS or PNP did not alter base-line monocyte adherence.
‡ TB plasma: TB plasma 5% by volume, not heat-inactivated replaced FCS in cell suspension.
§ 0.03 μg/ml AG added to PBMC in RPMI + 5% FCS.

TABLE IV
Effect of AG on PBMC Production of PGE_2

Experiment	PGE_2 production	
	Control*	3.0 μg/ml AG‡
	ng/ml/10^6 cells	
1	5	79
2	6	35
3	15	35
4	6	23
5	5	23
Mean±SEM	7±2	39±10

* 5×10^6 PBMC in 1 ml RPMI 1640 + 10% PHS were incubated 18 h in 5% CO_2 at 37°C.
‡ PGE_2 content of supernatant fractions was determined by radioimmunoassay.

saccharides was added 10% by volume to cell suspensions containing TB plasma or AG. In three experiments, the increases of monocyte attachment by TB plasma or AG were blocked completely by this antibody (Table V, $P < 0.02$). The immunoglobulin fraction of normal goat serum did not decrease the effect of AG on monocyte attachment.

Depletion of mycobacterial polysaccharide activity from TB plasma by affinity chromatography. To absorb mycobacterial polysaccharide from TB plasma, aliquots were eluted through immunoabsorbent columns of Sepharose 4B reacted with the antibody to mycobacterial polysaccharide. Aliquots of PNP were passed through identical immunoabsorbent columns to control for nonspecific effects of affinity chromatography on other plasma constituents. Affinity chromatography did not alter the titer of free AG antibody in TB plasma as determined by an enzyme-linked immunoassay. We then studied the effects of these column effluents of TB plasma and PNP on stimulated DNA synthesis and monocyte attachment to plastic.

TABLE V
Effect of Antibody to Mycobacterial Polysaccharide on the Augmentation of Monocyte Adherence to Plastic Induced by TB Plasma and AG

Addition to PBMC	Cells attached per well±SEM ($\times 10^{-3}$)
TB plasma	69±2.0
TB plasma + antibody to polysaccharide	55±1.0
AG	68±1.0
AG + antibody to polysaccharide	54±1.0
AG + NGS*	66±1.0

* NGS, immunoglobulin fraction of normal goat serum.

In the [^{3}H]thymidine incorporation assay, the column effluent of PNP or TB plasma was substituted as the protein source in the cell suspensions. However, neither column-passed plasma source adequately supported cell cultures. Therefore, the effect of column-passed TB plasma diluted 50% with PHS was examined. In four experiments, the mean PBMC response to PHA in cell cultures supplemented with PHS-diluted, column-passed TB plasma 44,301±6,317 cpm did not differ from the PHA response in PHS-supplemented cultures 49,810±7,433 cpm. In these experiments, TB plasma diluted 50% with PHS suppressed the PBMC response to PHA by 30±5% ($P < 0.002$).

In the quantative assay of monocyte attachment to plastic, PNP passed through this affinity column did not alter basal monocyte adherence (Table VI). While TB plasma before such manipulation increased monocyte adherence, the TB plasma passed through this affinity column did not increase monocyte adherence from control levels.

These observations demonstrated that passage of TB plasma over the immunoabsorbent containing antibody to mycobacterial polysaccharide depleted the suppressor and monocyte adherence-augmenting activity.

DISCUSSION

In various chronic infections of man, circulating suppressor cells (1–9), and immunosuppressive plasma (15–17), and/or parasite factors (12–14) have been defined. Our studies provide the first link among these observations. Both TB plasma and AG suppress PPD-induced [^{3}H]thymidine incorporation in PBMC from

TABLE VI
*Decreased Monocyte Activation by TB Plasma after Passage Through Affinity Column Containing Antibody to Mycobacterial Polysaccharide**

Addition to PBMC	Cells attached per well±SEM (10^{-3})
Nil	53±1.0
TB plasma	67±2.0
TB plasma—column passed‡	55±2.0
PNP§	51±2.0
PNP—column passed‡	53±1.0

* Affinity column prepared by reacting cyanogen-bromide activated Sepharose 4B with antibody to mycobacterial polysaccharide.
‡ TB plasma or PNP were passed in 1-ml aliquots through the affinity column each with a 5.0-ml bed volume.
§ Pooled plasma from 10 normal donors.

healthy subjects; suppression in each case is monocyte dependent and indomethacin reversible. TB plasma and AG have a direct inhibitory effect on T lymphocyte responses to PHA in addition to an indomethacin-reversible, presumably monocyte-mediated activity. The effects of TB plasma and AG were not due to cytotoxicity or altered kinetics of stimulated DNA synthesis, nor did these stimuli generate autonomously functioning suppressor cells; their action was reversible with washing.

Prostaglandin-secreting suppressor cells inhibit the response of T lymphocytes from healthy subjects to mitogens (10); immunosuppressive prostaglandins, in turn, act through a lymphocyte population that is either labile in culture or in its sensitivity to prostaglandin (31). In Hodgkin's disease, increased production of PGE_2 by adherent cells may provide the basis for their nonspecific suppression of lymphocyte responses (11). The PGE_2 production by normal monocytes is increased by in vitro exposure to such stimuli as bacterial lipopolysaccharide (32) and aggregated IgG (33). We excluded significant endotoxin contamination of our AG preparation using a limulus lysate assay. The inhibition of PBMC response by TB plasma and AG resembles prostaglandin-mediated suppression in its dependence on adherent cells, reversibility with indomethacin, and diminished effect on precultured cells. Moreover, we have demonstrated that culture of PBMC with AG stimulates production of immunoreactive PGE_2.

We also evaluated the effects of TB plasma and AG on quantitative attachment to plastic, a cell surface property augmented by stimuli increasing thromboxane A_2 production (34). Both TB plasma and AG increased monocyte adherence; their action was reversible with indomethacin. Thus, TB plasma and AG, by inference, lead to enhanced production of thromboxane A_2, as well as dienoic prostaglandin.

Passage of TB plasma over an immunoabsorbent column containing goat antibody to mycobacterial polysaccharide depleted the suppressive and monocyte adherence-augmenting factor(s). In addition, we found that the adherence-augmenting activity of TB plasma was reversible with this antibody to mycobacterial polysaccharides. Because the TB plasma contains IgG and IgM antibody to AG in high titer, polysaccharide-containing immune complexes may be responsible for such activity. The immunosuppressive properties of circulating immune complexes could derive from their immunoglobulin content, an attractive possibility because aggregated IgG increases monocyte production of PGE_2 (33). An alternate, more likely explanation, consistent with the blocking effect of antipolysaccharide antibody on monocyte adherence, is that exposed portions of the mycobacterial polysaccharides are biologically active. The demonstrated fivefold increase in mononuclear cell production of PGE_2 in the presence of AG and the immunosuppressive properties of AG also favor this interpretation. The extraordinary potency of AG that suppresses PBMC responses at 30 ng/ml, in fact, suggests that such polysaccharide products may be active in vivo. The absence of demonstrable pentose in tuberculous plasma in no way militates against this possibility in view of the relative insensitivity of the orcinol method (>2,000 ng/ml). In chronic mucocutaneous candidiasis, circulating yeast mannan has been ascribed a suppressor role (12). It is possible that microbial polysaccharides circulating alone or in immune complexes contribute to lymphocyte hyporesponsiveness in other chronic infections.

The precise role and importance of prostaglandins in the action of suppressor monocytes generated by diseases is uncertain. Although indomethacin reversed the suppressor cell activity demonstrated in Hodgkin's disease, there was no effect of indomethacin on monocyte-mediated suppression in tuberculosis (5) and only a small, though significant reversal of suppression in sarcoidosis (35). The mechanism of action of suppressor cells generated during tuberculosis is unknown. Studies in mice suggest that elicited/stimulated peritoneal macrophages produce less prostaglandin than do resident macrophages (36). Thus, "activation" of macrophages in vivo may be associated with a progressive decline in their capacity to produce prostaglandins. Furthermore, indomethacin reversibility does not establish whether an effect is mediated or modulated by prostaglandins. For example, in stimulated guinea pig macrophages, PGE_2 augments collagenase production by increasing cyclic AMP; the inhibition of collagenase production by indomethacin can be overcome by other stimuli which raise cyclic AMP levels (37). One possibility is that an initial prostaglandin-dependent, reversible process becomes prostaglandin-independent and irreversible during the course of tuberculosis.

In these studies, prostaglandin-secreting monocytes do not explain fully the suppressive activity of TB plasma and AG; a direct inhibition of the T lymphocyte response to PHA was demonstrated. In tuberculosis, T lymphocyte hyporesponsiveness affects responses not only to PPD but also to unrelated antigens (5); removal of suppressor monocytes restores only the PPD response. Thus nonspecific anergy in tuberculosis may be due to the direct effect of TB plasma or AG on T lymphocytes. Alternatively, these considerations suggest that the antigen-specificity of adherent cell function in tuberculosis and other chronic infections (1–4) may derive from monocyte-T lymphocyte interactions. In disease, the T lymphocyte suppressor

subpopulation may include a cohort of antigen-specific suppressor cells not present in the PBMC of our healthy donors. In tuberculosis, the effects of circulating mycobacterial polysaccharide on monocyte function may assume antigen specificity through such T lymphocyte suppressor cells. Our demonstration of the potent effects of TB plasma and AG on the mononuclear cell function of healthy subjects is the first step in testing this hypothesis.

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