BACILLE CALMETTE-GUÉRIN INFECTION IN THE MOUSE

Regulation of Macrophage Plasminogen Activator by T Lymphocytes and Specific Antigen*

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Experimental tuberculosis has been widely used to study cell-mediated immunity to infection (1–3) and the regulation of delayed type hypersensitivity (DTH)[1] to tuberculin and other antigens (4, 5). After infection with Bacille-Calmette-Guèrin (BCG), the host may acquire immunity to specific secondary challenge, protection against unrelated virulent organisms such as *Listeria monocytogenes* (6, 7), and also increased resistance to transplantable tumors (8). The macrophages become activated, display increased spreading and metabolic activity in culture, and may develop an enhanced capacity to kill specific as well as unrelated microorganisms. One pathway for activating macrophages depends on the generation of specifically sensitized thumus-derived (T) lymphocytes (9, 10), but little is known about the mechanism of activation or the nature and control of interactions among T cells, antigen, and macrophages.

We have reported previously that macrophages activated by various inflammatory and endocytic stimuli produce and secrete high levels of neutral protease activities including plasminogen activator (PA; 11, 12), collagenase (13), and elastase (14). Because the PA provides a sensitive measure of nonspecific macrophage activation and because antimicrobial activity of macrophages against slow growing and resistant organisms such as the tubercle bacillus is difficult to study (15), we asked whether macrophage PA could also be induced specifically, by infection with BCG.

In this report we describe the effects of systemic BCG infection in the mouse on PA activity of the peritoneal macrophages and examine the role of sensitized lymphocytes and of challenge with purified protein derivative (PPD) in regulating enzyme activity. Related studies on *Trypanosoma cruzi* infection in the mouse have been reported (16, 17).

Materials and Methods

Animals. NCS female mice, weighing 25–30 g, from The Rockefeller University were used in all studies except where noted. Inbred strains BALB/cJ and C_3H/HeJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

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[1] *Abbreviations used in this paper:* BCG, Bacille Calmette-Guérin; CF, culture filtrate, *Mycobacterium tuberculosis*, strain $H_{37}Rv$; DTH, delayed type hypersensitivity; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; IPA, inducer of plasminogen activator; MIF, migration inhibition factor; PA, plasminogen activator; PBS, phosphate-buffered saline; PPD, purified protein derivative; T, thymus-derived.

BCG Infection. Mice were infected intravenously (10.1 ml) with 2–6 × 10^7 viable BCG (strain 1011, Trudeau Institute, Saranac Lake, N. Y.; 18). Animals were kept six mice per cage, and controls were kept under the same conditions for the same period of time. BCG was stored at −70°C and thawed once before use.

Peritoneal cells were harvested from infected or control mice at different times after infection, with or without an intraperitoneal challenge with one of the following agents 2 days before harvest: (*a*) *Mycobacterium tuberculosis*, strain $H_{37}Ra$ (lyophilized; Difco Laboratories, Detroit, Mich.). Stock suspensions were sonicated 2 mg/ml in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin and 500 μg in 1 ml PBS injected per mouse. (*b*) *M. tuberculosis*, strain $H_{37}Rv$, culture filtrate (CF; 19). This was a gift from Professor M. Chase, The Rockefeller University, and was injected (0.01–10 μg) in a solution of PBS containing 5 μg/ml Tween 80 (ICI United States, Inc., Wilmington, Del.). (*c*) Tuberculin PPD (lot no. 16, Connaught Laboratories, Toronto, Canada), 50 μg in 1 ml PBS. (*d*) Proteose peptone (Difco Laboratories), 1 ml of a 1% solution.

Cells. For the purpose of this report cells obtained from uninfected control animals without intraperitoneal challenge are termed "resident cells." Peritoneal cells were harvested by conventional procedures and were usually pooled from three to four mice. Cells were washed and suspended in culture medium consisting of Dulbecco's medium (H-21; Grand Island Biological Co., Grand Island, N. Y.) supplemented with 2% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10^{-5} M mercaptoethanol (D2FBS). Cell yields from BCG-infected animals were 5–9 × 10^6 and contained 40–60% macrophages, with the remainder lymphocytes. Uninfected animals gave similar cell yields with 25–40% macrophages. Cell suspensions were distributed in multiwell Linbro plates (Linbro Chemical Co., New Haven, Conn.) with or without ^{125}I-fibrin. Unless noted otherwise each well contained 1 × 10^6 peritoneal cells of which 3–5 × 10^5 were macrophages.

Peritoneal cell cultures were either left unwashed and incubated further, with or without PPD, or were incubated for 2–4 h at 37°C and washed twice with Hanks' Balanced Salt Solution (HBSS) to remove most of the lymphocytes. These macrophage monolayers were refed D2FBS and used as described below.

Higher cell yields were obtained from infected animals challenged in vivo with the various agents listed above, 8–15 × 10^6 cells per mouse, with 5–20% polymorphonuclear luekocytes, 30–60% macrophages, and 30–50% lymphocytes. For these experiments, serial dilutions of cells were cultivated on ^{125}I-fibrin in Dulbecco's medium, antibiotics, and 20% FBS (D20FBS), with 60 μg/ml soybean trypsin inhibitor (STI fraction V, Miles Laboratories Inc., Elkhart, Ind.), to suppress fibrinolysis. The adherent cells were washed well, refed after 2–4 h, and incubated further in the same medium for at least 24 h to ensure loss of polymorphonuclear leukocytes (12).

Nylon-Wool Separation of Lymphocytes. Suspensions of peritoneal cells, 2–4 × 10^7 cells in 1–2 ml, were obtained in Dulbecco's medium + 10% heat-inactivated FBS + 5 × 10^{-5} mercaptoethanol (D10FBS) and overlayed on a nylon-wool column (300 mg); Fenwal Laboratories Inc., Morton Grove, Ill.) and preincubated with D10FBS at 37°C for 1 h (20). The column with cells was incubated at 37°C for 45 min, and the nonadherent cells eluted with 30 ml of D10FBS. The cells were washed once, counted, suspended to the desired concentration in D2FBS, and plated on radioactive Linbro plates containing a freshly plated monolayer of resident macrophages, with or without PPD. 10–25% of the peritoneal cells were recovered after passage over nylon-wool columns, of which fewer than 1% were macrophages. Cell viability, by trypan blue exclusion, was greater than 97%.

Nonadherent Cells. BCG-primed peritoneal cells were incubated in D10FBS for 1 h at 37°C, 1 × 10^7 cells per 100-mm tissue culture dish (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). The nonadherent cells were removed and pooled with cells removed by two gentle washes of the adherent monolayer. Cells were collected by centrifugation, resuspended in D2FBS, and added to monolayers of resident macrophages on ^{125}I-fibrin plates. 10–20% of peritoneal cells were recovered by this procedure with fewer than 5% macrophages.

Anti-Thy 1.2 Treatment. AKR anti-ASL_1 (anti-thy 1.2) serum was kindly provided by Dr. U. Hämmerling of the Sloan-Kettering Institute, N. Y. Peritoneal cells were obtained from four to six Balb/cJ or C_3H/HeJ mice 21–25 days after BCG infection. After washing, the cells were resuspended in RPMI-1640 + 1% FBS, 2 × 10^7/ml, divided into equal groups, and treated with antiserum or normal mouse serum (1/30 vol/vol), with or without selected rabbit serum (1/30 vol/

vol) as source of complement, and a trace amount of deoxyribonuclease. Each group of cells was incubated at 37°C for 45 min, washed, and resuspended to the same volume in D2FBS. The treated cells were then incubated in ^{125}I-fibrin plates for 1–2 days, with or without PPD, before assay. Treatment with anti-thy 1.2 serum and complement killed 24–31% of the peritoneal cells, as measured by trypan blue exclusion, and 7–16% of the cells were killed by either reagent alone.

Fibrinolysis. Fibrinolytic activity was measured either by cultivating cells directly on ^{125}I-fibrin or by a two-stage assay in which supernates, prepared by incubating cells with antigen, were added to monolayers of resident macrophages on ^{125}I-fibrin.

Direct Assay. Peritoneal cells were plated on ^{125}I-fibrin as described above and incubated at 37°C for 1–2 days with the desired concentration of PPD before assay.

Indirect Assay

PREPARATION OF SUPERNATE. Control and BCG-primed peritoneal cells were incubated in D2FBS with or without 1–50 μg PPD for 1–3 days at 37°C. Supernates were collected, centrifuged at 250 *g* for 15 min, and passed through a 0.45-μm Millex filter (Millipore Corp., Bedford, Mass.). Supernates were stable during storage at −70°C.

INDUCTION OF FIBRINOLYSIS. Resident peritoneal cells, containing 3–5 × 10^5 macrophages per well, were cultivated on ^{125}I-fibrin in D2FBS for 2 h at 37°C. The macrophage monolayers were washed twice with HBSS and incubated for 1–3 days with supernates diluted in D2FBS before assay.

^{125}I-fibrin-coated Linbro plates were prepared as described (21). Each well contained 20 μg fibrinogen and 1 × 10^5 cpm ^{125}I releasable by trypsin. Assays were run in duplicate, and appropriate controls were included in all experiments. Media were monitored for release of radioactivity before each change of medium. The assay of fibrinolysis was started by washing cells twice in HBSS followed by incubation in 0.5 ml Dulbecco's medium containing 5% dog serum which had been acid-treated to destroy inhibitors (pH 2/30 min at room temperature). 100-μl aliquots of medium were withdrawn at intervals (1–6 h) and assayed for release of radioactivity in a Packard gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Under these conditions, fibrinolysis is strictly dependent on the presence of plasminogen and is therefore due to macrophage PA.

Lysozyme activity was measured as described (22).

Reagents. FBS and dog serum were obtained from Grand Island Biological Co. Endotoxin (*Salmonella abortus equi*) was a gift from Professor O. Westphal, Freiburg, Germany. Plasminogen was purified from dog serum by lysine-sepharose chromatography (23).

Results

BCG Infection and Challenge with Antigen in Vivo. The influence of systemic infection and intraperitoneal challenge with various antigenic stimuli on macrophage fibrinolysis was first examined. Peritoneal macrophages obtained from animals 3 wk after infection with 2–6 × 10^7 viable organisms spread rapidly in culture, but showed only a modest, twofold increase in fibrinolytic activity (Table I). Higher levels of macrophage fibrinolysis were observed when BCG-primed animals were challenged with specific antigen 2 days before harvest. Two soluble antigen preparations, PPD and *M. tuberculosis* CF, further enhanced activity fourfold in infected animals. A particulate antigen, $H_{37}Ra$, also resulted in a significant increase in uninfected controls, perhaps the result of phagocytic stimulation (12). Proteose peptone provided a less effective, but definite, nonspecific stimulus in infected animals. Inasmuch as nonspecific challenge with these antigens did not enhance macrophage fibrinolysis to the same extent, it seemed likely that PA was induced by an immunologically specific mechanism.

In Vitro Challenge of BCG-Primed Peritoneal Cells. We next sought conditions to provide a secondary stimulus with specific antigen in vitro.

TABLE I

*Effect of Intraperitoneal Challenge on the Fibrinolytic Activity of Cultivated Macrophages from Mice Infected with BCG**

Treatment		PA
Infection	Challenge	
		% radioactivity released/4 h/10^6 cells
Control	Nil	2.0
	PP	1.5
	$H_{37}Ra$	6.1
	CF	1.5
	PPD	2.5
BCG	Nil	4.5
	PP	7.5
	$H_{37}Ra$	13
	CF	15
	PPD	18

* Mice were infected intravenously with 2–6 × 10^7 viable BCG. After 3 wk the animals were injected intraperitoneally with 1 ml of a 1% solution of proteose peptone (PP), 500 μg $H_{37}Ra$, 0.1 μg CF or 50 μg PPD. Peritoneal cells were harvested after 2 days and were cultivated in D_{20}STI for 24 h before assay.

Peritoneal cells were obtained from mice infected 3 wk earlier with BCG, and the unfractionated population of macrophages and lymphocytes incubated on ^{125}I-fibrin with different amounts of PPD for 1 or 2 days. The cultures were then washed to remove nonadherent cells and the fibrinolytic activity of the macrophage monolayer determined. As shown in Fig. 1, the addition of PPD to BCG-primed cultures resulted in a striking dose-related enhancement of macrophage fibrinolytic activity. Stimulation of fibrinolysis was fully evident within 1 day of incubating peritoneal cells with PPD and was maximal, in this experiment, at a concentration of 2 μg/ml. A higher concentration of PPD, up to 50 μg/ml, was required for maximal fibrinolysis with cells from some groups of infected animals, and the optimal concentration of PPD was determined by titration in all subsequent experiments.

Control experiments showed that two different preparations of antigen, PPD and *M. tuberculosis* CF, could both be used for challenge in vitro and that purified endotoxin, an inducer of macrophage PA in vivo (12), did not stimulate fibrinolysis by BCG-primed cells in culture. The fibrinolysis observed after PPD stimulation was strictly dependent on plasminogen and the presence of macrophages.

From these experiments, we concluded the exposure of BCG-primed peritoneal cells to PPD in vitro provided an effective secondary stimulus for induction of macrophage PA.

Time-Course of Infection. We next examined the effect of duration of BCG infection on the enhancement of macrophage fibrinolysis by in vitro challenge with PPD. As shown in Fig. 2 b, enhancement of macrophage fibrinolysis by PPD was detectable within 2 wk, became maximal after 3 wk, and declined

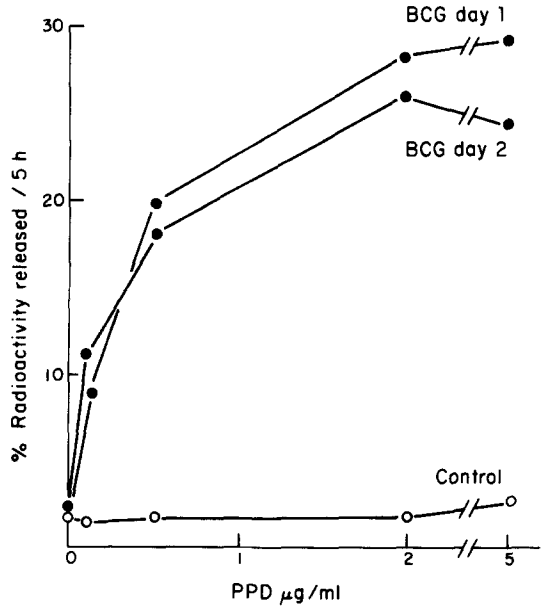

FIG. 1. Enhanced macrophage fibrinolysis after challenge of BCG-primed cells with PPD in vitro. Peritoneal cells were harvested 26 days after infection with BCG or from control, uninfected animals. Cells were cultivated for 1 or 2 days on ^{125}I-labeled fibrin plates in D2FBS with or without PPD, washed, and the resulting macrophage monolayer assayed for fibrinolytic activity.

subsequently. Enlargement of spleen (Fig. 2 a) and liver followed a roughly similar time-course, but was noted earlier and diminished more slowly with time. Fig. 2 c shows further that the three to fourfold stimulation of fibrinolysis by PPD was selective, whereas lysozyme secretion by the same macrophages was unaffected.

Sensitized Lymphocytes and PPD Induce Fibrinolysis in Resident Macrophages. To learn more about the nature of the antigen-sensitive cells, we next separated the lymphoid and macrophage elements of the BCG-primed peritoneal cavity. Cells were passed over nylon-wool columns to remove macrophages, and nonadherent lymphocytes were then co-cultivated with control resident macrophages with or without PPD. After incubation for 1 or 2 days, the lymphocytes were removed by washing, and macrophage fibrinolysis assayed. Figs. 3 and 4 illustrate experiments in which nylon wool purified sensitized lymphocytes and PPD stimulated fibrinolysis by resident macrophages approximately fourfold, whereas lymphocytes from uninfected animals had no such activity. In control experiments, not shown, BCG-primed lymphocytes displayed no fibrinolytic activity in the absence of macrophages and died within 1–2 days in culture.

Induction of fibrinolysis in resident macrophages was proportional to the dose of PPD (Fig. 3) and to the number of sensitized lymphocytes (Fig. 4). The optimal concentration of PPD varied from 5 to 50 μg/ml, and enhanced fibrinolysis could be detected at a lymphocyte: macrophage ratio of 1:40, although maximal stimulation was observed at a ratio of 1:1. Co-cultivation of

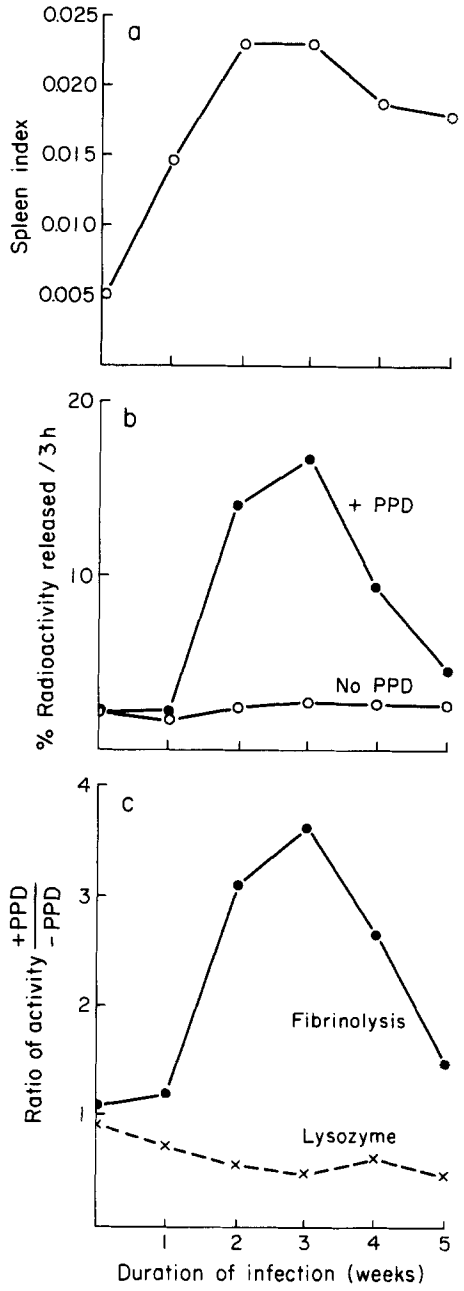

FIG. 2. Effect of duration of BCG infection on enhancement of macrophage fibrinolysis by PPD. Groups of mice were infected at weekly intervals with BCG, 2-6 × 10^7 viable organisms, intravenously. Peritoneal cells were cultivated on ^{125}I-fibrin plates, 1 × 10^6 cells/well, in the presence or absence of 10 μg/ml PPD, for 1 day. Cultures were then washed, and macrophage fibrinolysis and lysozyme secretion determined. (a) Spleen index; (b) fibrinolysis; (c) ratio of fibrinolysis and lysozyme secretion with and without PPD.

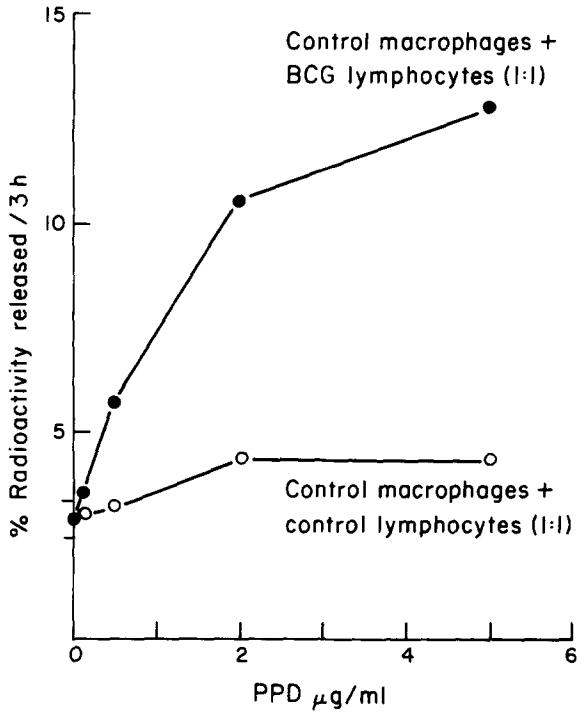

FIG. 3. Stimulation of fibrinolysis in resident macrophages by BCG-primed lymphocytes and PPD. Nylon-wool-separated lymphocytes were prepared 26 days after infection or from control, uninfected animals. 4×10^5 lymphocytes were added to ^{125}I-fibrin-coated plates containing 4×10^5 resident macrophages/well and incubated in D2FBS with different concentrations of PPD, for 2 days, before assay.

sensitized lymphocytes and resident macrophages with PPD for 1 or 2 days gave similar results.

The enrichment of antigen-sensitive lymphocytes in BCG-primed cell populations is shown in Table II and compares the induction of macrophage fibrinolysis by unfractionated peritoneal cells, nylon-wool separated lymphocytes, and lymphocytes separated by nonadherence to a tissue culture dish. Cell recovery for nylon-wool separated and nonadherent lymphocytes was 11 and 6%, but recovery of PPD-dependent induction of fibrinolysis was 33 and 27% for each fraction, indicating a possible enrichment of three- to fourfold in specific activity. This estimate is approximate because of the intrinsic activity of macrophages in the various fractions.

Role of Thymus-Derived (T) Lymphocytes. Induction of macrophage fibrinolysis by infection with *T. cruzi* (17) requires T lymphocytes. To establish whether T cells also play a part in BCG infection, peritoneal cells obtained 3 wk after infection were exposed to anti-thy 1.2 antiserum with or without complement and then incubated in the presence or absence of PPD for 1 day before assay. As shown in Table III, pretreatment with anti-thy 1.2 antiserum with complement prevented the PPD-dependent stimulation of macrophage fibrinolysis, whereas treatment with either reagent alone resulted in three- to fivefold enhancement of activity. The low levels of PPD-independent activity were

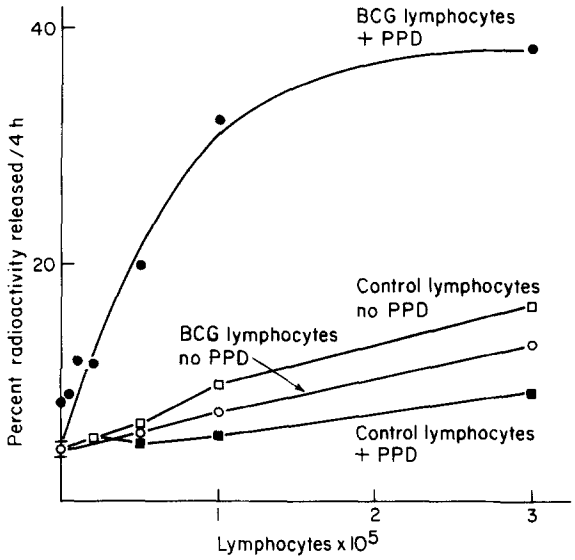

FIG. 4. Dose response of stimulation of macrophage fibrinolysis by BCG-primed lymphocytes with PPD. Nylon-wool-separated lymphocytes were obtained from control, uninfected animals or 24 days after BCG infection. Suspensions of lymphocytes were added to monolayers containing 3×10^5 resident macrophages/well and cultivated in D2FBS, with or without 20 μg PPD, for 2 days, before assay. (●—●) BCG lymphocytes with PPD: (○—○) BCG lymphocytes without PPD; (■—■) control lymphocytes with PPD; (□—□) control lymphocytes without PPD.

not influenced. Induction of macrophage fibrinolysis by PPD in BCG infection therefore depends on sensitized T lymphocytes.

Supernatant Inducer of Macrophage PA. Lymphoid cultures release soluble mediators which enhance macrophage fibrinolysis after sensitization and specific challenge (17) or after lectin stimulation (24, 25). Peritoneal cells from BCG-infected and control animals were therefore incubated with PPD, and the supernate added to resident macrophages. Fig. 5 a illustrates an experiment in which supernate from BCG-primed cells challenged with PPD enhanced macrophage fibrinolysis more than fourfold, whereas control supernate or medium showed no such effect. A dose-response experiment (Fig. 5 b) showed that stimulation of fibrinolysis was maximal at a supernatant concentration of 25–50%, but was detectable at 2.5% vol/vol.

Because the fibrinolysis was dependent on plasminogen, we postulate that the active supernate contained an inducer of PA (IPA). Production of IPA was detectable within 1 day of antigen challenge, but increased with time and was maximal after 2–3 days. Optimal levels of IPA were produced at low concentrations of serum, 1–5% FBS or 1–10% horse serum. Higher concentrations of FBS were strongly inhibitory. Heat inactivation of serum (56°C/30 min) had no effect nor did the addition of mercaptoethanol. Although sensitized cells yielded the highest levels of IPA activity after challenge with PPD, some preparations contained low but significant levels of activity without PPD. Spleen cultures from BCG-infected animals also generated IPA after stimulation with PPD.

The effects of supernate containing IPA on resident macrophages were also

TABLE II
*Stimulation of Fibrinolysis of Resident Macrophages by BCG-Primed Peritoneal Cells and PPD**

Treatment	Cell yield 10^5/mouse	Macrophage fibrinolysis			
		−PPD		+PPD	
		sp act U/10^5 cells	*yield* U/mouse	*sp act* U/10^5 cells	*yield* U/mouse
Peritoneal cells	61	0	0	1.9‡	12
Nonadherent	3.6	0.60	0.22	9.0	3.2
Nylon wool	6.6	0.50	0.33	6.0	4.0

* Peritoneal cells were harvested from mice 22 days after infection with BCG. Serial dilutions of total peritoneal cells, nonadherent and nylon-wool-separated lymphocytes were added to monolayers of resident macrophages with or without PPD (10 μg/ml) and cultivated for 2 days before assay.

‡ Fibrinolytic activity of peritoneal cells without resident macrophages subtracted (1.7 U/1 × 10^5 cells).

TABLE III
*Effect of Anti-Thy 1.2 Antiserum and Complement on Stimulation of Macrophage Fibrinolysis by BCG Infection and PPD**

Treatment	Macrophage fibrinolysis	
	−PPD	+PPD
	% radioactivity released/4 h	
Anti-thy 1.2	3.4	19
Normal mouse serum + rabbit complement	4.9	15
Anti-thy 1.2 + rabbit complement	4.5	5.7

* Peritoneal cells were obtained from six male Balb/c mice 21 days after infection with BCG. After treatment with antiserum and complement (see Materials and Methods), 4 × 10^5 cells were incubated on ^{125}I-fibrin with or without 50 μg PPD for 1 day before assay.

studied. Enhanced fibrinolysis was noted after 1 day, but increased with duration of exposure and was maximal at 3 days. Macrophages that had been exposed to active supernates spread more extensively than controls, showed active membrane ruffling, and accumulated numerous phase-dense granules in the perinuclear region. Such activated resident macrophages did not show the toxicity sometimes seen with macrophages from BCG-infected animals after PPD challenge in the presence of sensitized lymphocytes. Lysozyme secretion by macrophages was not enhanced by exposure to supernates rich in IPA (not shown).

Discussion

BCG infection in the mouse elicits a variety of humoral and cellular responses, among which effects on lymphocyte and macrophage function feature prominently (1–5). The present studies show that high levels of macrophage

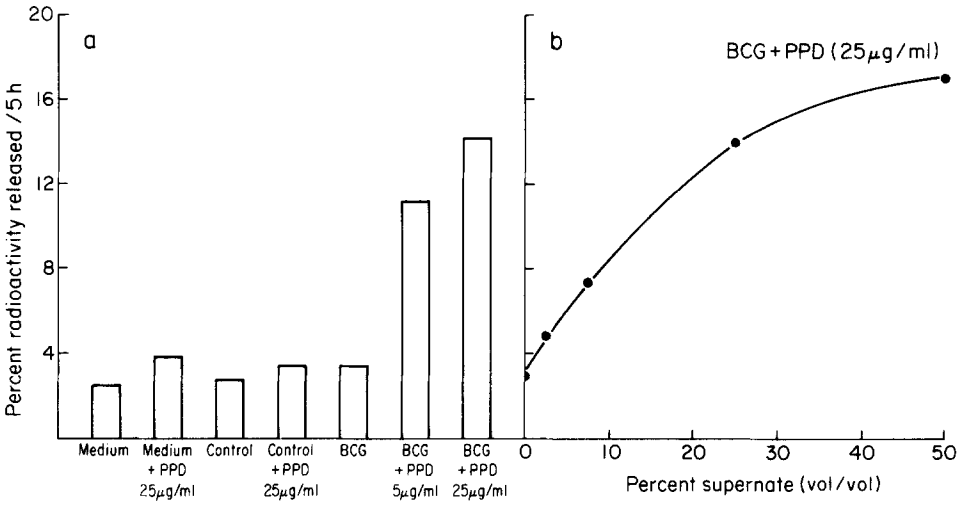

FIG. 5. Induction of macrophage fibrinolysis by peritoneal cell supernates after BCG infection and PPD. Cells obtained from control animals or 30 days after infection with BCG were cultivated for 2 days on nonradioactive Linbro plates 1×10^6 cells/well, in D2FBS, with or without PPD. Supernates were then added at different concentrations to resident macrophages on ^{125}I-fibrin plates, 4×10^5/well, for 3 days before assay. (a) Macrophage fibrinolysis, concentration of supernates 25% vol/vol; (b) dose response of supernate prepared from BCG-primed cells after challenge with 25 μg/ml PPD.

fibrinolysis are induced under conditions that parallel the expression of cell-mediated immunity and DTH (26). Enhanced fibrinolysis can be elicited by specific challenge of BCG-primed animals in vivo or of sensitized peritoneal cells directly in culture. The antigen-sensitive response depends on T-lymphocytes that may stimulate fibrinolysis by macrophages from uninfected and infected animals either after co-cultivation or by the release of soluble mediators. Enhanced fibrinolysis is due to macrophage PA and is regulated by an immunologically specific mechanism, as shown by these and related studies with *T. cruzi*. Although highest levels of macrophage fibrinolysis were observed when BCG- and *T. cruzi*-immune animals were challenged with specific antigen, nonspecific stimulation of PA may follow direct challenge of primed macrophages with a particulate stimulus. The present system offers the advantage of a soluble antigen. Further evidence for immunologically specific regulation of macrophage PA has been provided by studies of mixed leukocyte culture reactions in the mouse[2] and of hypersensitivity to PPD in guinea pigs immunized with Freund's adjuvant (25).

Our results draw attention to the role of antigen restimulation and of T cells in control of macrophage activation. No significant fibrinolysis was found without secondary challenge, although the peritoneal cavity of BCG-infected mice contained substantial numbers of sensitized T cells, as noted previously by others (27). These sensitized lymphocytes responded rapidly to PPD, and measurement of macrophage fibrinolysis provided a sensitive and quantitative

[2] Newman, W., S. Gordon, U. Hämmerling, and B. R. Bloom. Manuscript submitted for publication.

measure of their generation in the peritoneal cavity during infection. In the course of infection, the sensitization of peritoneal lymphocytes becomes apparent somewhat later and declines more rapidly than enlargement of the spleen and liver. It is possible that sensitized cells persist longer in the spleens of BCG-infected animals than after infection with *T. cruzi* (17). The decline of PPD-stimulated fibrinolysis by peritoneal cells at later stages of infection could be due to either loss or inhibition of sensitized T cells.

Induction of macrophage PA by specific antigen depends on T lymphocytes in BCG infection, as with *T. cruzi* (17) and the mixed leukocyte culture reaction (see fn. 2). T cells are known to play a role in antimicrobial protection in BCG-infected mice (3, 27) and rats (28) and in development of DTH to tuberculin (29, 30). Nothing is known, however, about the frequency, heterogeneity, or proliferative characteristives of T cells that regulate macrophage fibrinolysis or their relation to protective and memory cells generated by BCG.

Stimulation of fibrinolysis by BCG infection provides a particularly useful model system to study interactions between sensitized lymphocytes, antigen, and macrophages under defined conditions in culture. Titration experiments with nylon-wool-separated lymphocytes and active supernates showed that direct contact and soluble mediators stimulated macrophage fibrinolysis to a similar extent. The 2-stage assay offers the possible advantage of separating production of a PA inducer from its action on macrophages, although it is likely that macrophages also play a role in secondary stimulation of sensitized T cells by antigen (31, 32). A particular advantage of the BCG model, however, is that sensitized lymphocytes and PPD can be titrated directly on macrophages from uninfected animals. The sensitivity of the fibrinolytic assay is greatly enhanced by serum-free conditions, and it may be possible to determine the activity and number of sensitized peritoneal lymphocytes by limiting dilution analysis.

The nature of the inducers of macrophage PA remains obscure, although it is known that lymphocytes release similar products after stimulation with lectins (24, 25). The IPA generated by MLC reactions closely resembles migration inhibition factor in activity, size, and in production by both Ly-1 and Ly-2,3 subsets of T lymphocytes (see fn. 2). Other workers have reported that PA produced by transformed fibroblasts is itself able to generate MIF-like activity from undefined serum components (33). The supernatant inducer of macrophage PA studied in the present report cannot be PA itself because no fibrinolytic activity could be detected before incubation with macrophages and because medium conditioned by thioglycolate broth-stimulated macrophages, a rich source of PA, did not enhance fibrinolysis by unstimulated macrophages. The relationship between IPA, MIF, PA, and serum zymogens clearly deserves further study.

Products of stimulated lymphocytes have several effects on macrophages other than stimulating PA and inhibiting migration (34). Responses of macrophages reported include enhanced secretion of collagenase (35), C_2 (36), and pyrogens (37), increased plasma membrane spreading, ingestion of EIgMC via receptors for C_3b,[3] and formation of giant cells (38). The present studies show

[3] Bianco, C., S. Gordon, and B. Bloom. Unpublished observations.

that lysozyme production is, however, not influenced. Purification of IPA is necessary to determine whether the multiple macrophage responses are mediated by a common molecular mediator.

Induction of macrophage PA by T cells provides an important mechanism to amplify lymphocyte function, to generate various mediators of inflammation, and may also contribute to connective tissue catabolism and tissue injury associated with DTH in tuberculosis. Activation of macrophages by this pathway can be augmented by endocytic stimuli and by humoral mediators generated by activation of complement and coagulation cascades (39). The mechanism by which BCG-activated macrophages could acquire antimicrobial and antitumor cell activity, however, remains unknown.

Summary

High levels of plasminogen activator (PA) were induced in mouse peritoneal macrophages by infection with BCG, 2–6 × 10^7 viable organisms intravenously, followed 3–4 wk later by intraperitoneal challenge with purified protein derivative (PPD) 2 days before harvest. Macrophages obtained from infected animals without boosting showed little fibrinolytic activity, but challenge of Bacille-Calmette-Guèrin (BCG)-primed peritoneal cells with PPD in culture also enhanced macrophage PA 4- to 10-fold. Stimulation of macrophage PA by PPD depended on specifically sensitized thymus-derived (T) lymphocytes because it was abolished by pretreatment of BCG-primed peritoneal cells with anti-thy 1.2 antiserum and complement. A direct assay was developed in which nylon wool separated sensitized lymphocytes and PPD induced PA in macrophages from uninfected animals under defined conditions on ^{125}I-fibrin. Enhanced macrophage fibrinolysis was proportional to concentration of PPD and the number of sensitized lymphocytes transferred. An indirect two-stage assay was also used to show that BCG-sensitized peritoneal cells released a soluble inducer of macrophage PA into the culture medium, after challenge with PPD.

Induction of macrophage PA by PPD challenge in vitro made it possible to study the generation and activity of sensitized peritoneal lymphocytes at different stages of infection. Our results show that nonadherent peritoneal cells of BCG-infected mice provide a rich source of specifically sensitized lymphocytes and that macrophage activation is limited by continued availability of antigen, as well as sensitized lymphocytes. Induction of macrophage PA provides a sensitive, versatile, and rapid in vitro assay to study the role of lymphocytes and specific antigen in macrophage activation by BCG.

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