Strain Variation of Bacillus Calmette–Guerin-Induced Pulmonary Granuloma Formation Is Correlated With Anergy and the Local Production of Migration Inhibition Factor and Interleukin 1

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Pulmonary granulomatous inflammation was induced by the intratracheal injection of viable bacillus Calmette–Guerin (BCG) into genetically high granuloma responder (C57BL/6J and BALB/c) and low responder (CBA/J) mice with and without immunization by methylated bovine serum albumin in complete Freund's adjuvant. Significant migration inhibition factor (MIF) and interleukin 1 (IL 1) activities were detected in aqueous lung granuloma extracts prepared from high responder mice bearing BCG-induced granulomatous inflammation. Interleukin 2 activity was not detected. Very low MIF and IL 1 activities were detected in extracts from low responder mice. Furthermore, high responder, but not low responder, mice showed marked suppression of *in vivo* and *in vitro* manifestations of cell-mediated immunity to both specific and nonspecific antigens. In contrast, humoral antibody response was not affected significantly. The kinetics of anergy in granuloma-bearing mice correlated closely with the appearance of MIF and IL 1 activities in the lesions. Thus, genetically determined granuloma response to BCG and the expression of anergy in various strains of mice were well associated with *in vivo* release of MIF and IL 1. These results indicate that the genetic ability or inability to mount a granulomatous inflammatory response to BCG may extend to the capacity of cells within the lesions to generate soluble mediator(s) which is also responsible for anergy in granuloma-bearing mice. (Am J Pathol 1985, 119: 223–235)

OUR PREVIOUS STUDIES have demonstrated that migration inhibition factor (MIF) and interleukin 1 (IL 1) activities are generated locally within the pulmonary granulomas that develop when sensitizing antigen-conjugated agarose beads are injected intratracheally into immunized guinea pigs and mice.[1,2] Furthermore, we have shown that the presence of active granulomatous inflammation in mice with suppression of *in vivo* and *in vitro* manifestations of cell-mediated immunity (anergy) is associated with the appearance of MIF and IL 1 activities in extracts prepared from inflamed lung. In addition, the anergic state in granuloma-bearing mice may be responsible for the impaired antigen-induced interleukin 2 (IL 2) production.[3] These studies clearly suggest that the lymphokines and interleukins might contribute to granuloma formation and the associated anergy.

The present study was conducted to determine whether the above findings extend to bacillus Calmette–Guerin (BCG)-elicited granulomatous inflammation in mice. We demonstrate that there is strain variation of BCG-induced granuloma formation in mice which correlates closely with the appearance of MIF and IL 1 activities in the granuloma extracts, and also that anergy develops in the high granuloma responder strain. The results obviously indicate that the genetic determination of granuloma response to BCG is expressed in the *in vivo* release or production of these mediators and factor(s) which is responsible for anergy. The results also support the concept that lymphokines and inter-

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leukins play a vital role in BCG-elicited granulomatous inflammation and the development of anergy.

Materials and Methods

Mice

Inbred female C57BL/6J and CBA/J mice were obtained from the Jackson Laboratory, Bar Harbor Maine. Female BALB/c mice were purchased from West Seneca Laboratory, Buffalo, New York. All mice were between 7 and 9 weeks of age at the time they were used.

Immunization

Mice were immunized by subcutaneous injection of 125 μg of methylated bovine serum albumin (MBSA; Sigma Chemical Company, St. Louis, Mo) emulsified in complete Freund's adjuvant into front footpads, inguinal regions, and the base of the tail. Complete Freund's adjuvant containing 2 mg of heat-killed *Mycobacterium tuberculosis* (H_{37} Rv, Aoyama strain) per milliliter of incomplete Freund's adjuvant (Difco Laboratories, Detroit, Mich). *M tuberculosis* was kindly supplied by the Department of Tuberculosis, NIH, Tokyo.

Induction of Pulmonary Granulomatous Inflammation by BCG

Ten days after immunization, the mice were injected intratracheally with a 0.1 ml suspension of viable BCG at the concentration of 2.5 mg/ml. As controls, nonimmunized mice were given injections of the same dose of BCG, and immunized mice were given injections of physiologic saline. BCG was obtained from the Department of Tuberculosis, NIH, Tokyo.

Histologic Examination

Routine paraffin-embedded hematoxylin and eosin (H&E)-stained sections were prepared from each excised lung and evaluated by light microscopy.

Aqueous Granuloma Extracts

Mice were killed at various intervals 1 to 21 days after intratracheal challenge with BCG. Aqueous extracts of the granuloma-bearing lungs were prepared by a modification of the method of Pellergrino and Brener,[4] as described previously.[2] Briefly, lungs inflated with RPMI 1640 (GIBCO, Grand Island, NY) were placed in a Waring blender containing 50 ml of RPMI 1640 medium per lung and ground for 30 seconds at low speed. Tissue was centrifuged at 200*g* for 5 minutes and resuspended in 5 ml of RPMI 1640 medium. The granuloma suspension was then homogenized with a Polytron (Brinkmann Instruments, Westbury, NY) for 30 seconds. During these procedures, tissue was kept on ice. The homogenized tissues were then centrifuged at 2000*g* for 30 minutes. Samples were then sterilized by filtration through a millipore membrane (pore size, 0.45 μ). The extract was aliquoted and stored at −70 C until use. Aqueous granuloma extracts contained 0.4–0.5 mg protein/ml of medium as determined by the method of Lowry et al.[5]

Indirect Murine MIF Agarose Assay

A modification of the agarose microdroplet assay originally reported by Harrington and Stastny[6] was used. Peritoneal exudate cells from syngeneic mice were induced by injection of 2 ml of light mineral oil intraperitoneally 4 days before collection. The cells were suspended at 1.5×10^8 cells/ml in RPMI 1640 containing 0.2% sea plaque agarose (Marine colloid, Rockland, Maine) and 10% heat-inactivated normal guinea pig serum. A 1-μl droplet of the cell mixture was placed in the center of each well of a 96-well microtiter culture plate (Falcon, Oxnard, Calif). After the droplet gelled, 100 μl of test sample was added to each well. After an 18–24-hour incubation, the migration was quantitated on an inverted light microscope equipped with a 0.5-sq mm reticule (Edmund Scientific, Barrington, NJ). The migration inhibition activity was calculated from four replicate samples as follows:

$$\%\ \text{migration inhibition} = \left(1 - \frac{\text{migration area in test sample}}{\text{migration area in control}}\right) \times 100$$

A migration inhibition of 20% or greater constituted significant MIF activity.

Determination of IL 1 Activity

IL 1 activity was measured by the enhancement of BALB/c thymocyte proliferative response to a suboptimal concentration of phytohemagglutinin-P (PHA-P; Difco) as described by Oppenheim et al.[7] Briefly, thymocytes from BALB/c mice were suspended at a density of 1×10^7 cells/ml in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 2.5×10^{-5} M 2-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin (referred below as complete medium). Thymocytes were cultured for 72 hours at 1×10^6 cells/well in the presence of 1 μg/ml PHA-P and serially diluted samples to be assayed for IL 1 activity. Cultures were pulsed with 0.2 μCi of ^{3}H-thymidine (^{3}H-TdR; specific activity 6.7 Ci/mmol, New

England Nuclear, Boston, Mass) for a final 16-hour incubation. Cultured thymocytes were harvested, and the ^{3}H-TdR incorporation was measured in a liquid scintillation counter. One IL 1 unit was defined as the amount of IL 1 required to double the proliferative response of thymocytes to the submitogenic concentration of PHA-P.[8] Units are expressed as the mean ± SEM in triplicate.

Assay for IL 2

Granuloma extracts were assayed for IL 2 activity on the basis of its ability to support proliferation of an IL 2-dependent murine cytotoxic T-lymphocyte line (CTLL) as described by Gillis et al.[9] The CTLL cells were generously provided by Dr. K. A. Smith, Department of Medicine, Dartmouth Medical School, Hanover, New Hampshire. Cells were washed and resuspended at a concentration of 5×10^4 cells/ml of complete medium. One hundred microliters of the cell suspension was cultured in each well of a microtiter plate with serial twofold dilutions of samples to be assayed. After 20 hours of incubation, 0.5 μCi of ^{3}H-TdR was added, and the incubation was resumed for an additional 4 hours. Values of ^{3}H-TdR uptake shown are the mean counts per minute ± SEM of triplicate determinations.

Neutralizing Effect of Granuloma Extracts on Preformed Murine IL 2

IL 2 activities were assayed in culture containing granuloma or control murine lung extracts (50 μl) and various dilutions of standard murine IL 2 preparations (final volume, 250 μl/well). Standard murine IL 2 was produced by culturing BALB/c spleen cells (5×10^6 cells/ml) with 5 μg/ml of concanavalin A (Con A; Sigma) for 48 hours. The IL 2-containing supernatant was supplemented with a final concentration of 10 mM methyl-α-D-mannopyranoside (Sigma) to inactivate residual Con A.

Footpad Test for Delayed-Type Hypersensitivity

At various intervals after intratracheal challenge of viable BCG, BALB/c and CBA/J mice were given injections in the hind footpads with 20 μl of 2 mg/ml purified protein derivative (PPD; Connaught Laboratories, Willowdale, Ontario, Canada) or 2 mg/ml MBSA in physiologic saline. Because C57BL/6J mice do not develop a footpad response to PPD,[10] we used 20 μl of BCG suspension (2.5 mg/ml) to test this strain of mice. The difference of footpad thickness at 24 hours after and just before challenge injection was measured. Triplicate measurements of 24-hour footpad swelling induced by antigenic challenge were made with an engineer's micrometer (Starret, Athol, Mass). The percentage of swelling was calculated as a ratio relative to immunized syngeneic control mice challenged intratracheally with saline.

Lymph Node Cell Proliferative Response to Antigen or Mitogen

A modification of the method described by Corradin et al[11] was used to assay for antigen- or mitogen-induced lymphocyte proliferation. The lymph node cell suspensions were prepared from animals at various times as indicated in each experiment. Axillary, brachial, and inguinal lymph nodes were removed, and single cell suspensions were prepared by gentle compressing and teasing of the node between the frosted ends of two glass slides. The procedure routinely provided single cell suspensions with 90–95% viability as assessed by trypan blue dye exclusion. Cells were suspended at a density of 1×10^6 cells/ml in the complete medium, and 0.2 ml of cell suspension was dispensed in a microculture plate. Antigen (50 μg/ml MBSA or 100 μg/ml PPD, optimal antigen concentration, as determined by dose-response studies) or 2.5 μg/ml PHA-P was added to triplicate cultures. Plates were incubated at 37 C for 4 days, and 0.2 μCi of ^{3}H-TdR was added to each well 16 hours before harvesting. Data were expressed as a "Δ cpm," representing the difference in uptake of ^{3}H-TdR between antigen- or PHA-P-stimulated and unstimulated controls.

Detection of Anti-MBSA-Specific Serum IgG Antibody

Antibodies to MBSA were measured by a modified solid phase ^{125}I-protein A radioimmunoassay described by Goding.[12] Briefly, each well of a flexible polyvinyl chloride plate (Dynatech Laboratories, Alexandria, Va) was coated with 5 mg/ml of MBSA by incubation for 2 hours at room temperature. After discarding of the MBSA solution, the wells were washed three times with borate-buffered saline (BBS), pH 8.0. Fifty microliters of a 1/10 dilution of sample serum was added to each well and incubated for 3 hours at room temperature. The wells were then washed four times with BBS, and 50 μl (4×10^4 cpm) of ^{125}I-protein A (specific activity, 70–100 μCi/μg, New England Nuclear) was added into each well. After 3 hours, incubation at room temperature, the wells were washed five times with BBS. Individual wells were cut apart, and the radioactivity was counted with a gamma-counter. Data represent the mean cpm ± SEM of triplicate determinations.

Results

Histologic Characteristics of the Lung

In C57BL/6J and BALB/c mice, histologic study of the lung showed severe, diffuse intraalveolar and interstitial inflammatory cell infiltration at early stage (1–3 days) after intratracheal viable BCG challenge. The infiltrate had a peribronchial distribution and was primarily composed of large macrophages, mature lymphocytes, and scattered neutrophils (Figures 1A and B). Inflammatory cell infiltration began approximately 6 hours after BCG challenge in these immunized mice and was more prominent in immunized than nonimmunized mice. In contrast, CBA/J mice, regardless of prior immunization, showed only a very mild interstitial mononuclear cell infiltration at early stage (Figure 1C). Two and 3 weeks following intratracheal BCG challenge, C57BL/6J and BALB/c mice, regardless of prior immunization, showed randomly distributed, well-organized granuloma composed of epithelioid macrophages, multinucleated giant cells, and lymphocytes (Figures 1D and E). On the other hand, CBA/J mice showed rare and smaller epithelioid granulomas at these later times (Figure 1F). These results indicate that C57BL/6J and BALB/c mice are high granuloma responder strains, whereas CBA/J mice are low granuloma producers in response to the intratracheal challenge of viable BCG. These results are consistent with those of previous reports.[10,13]

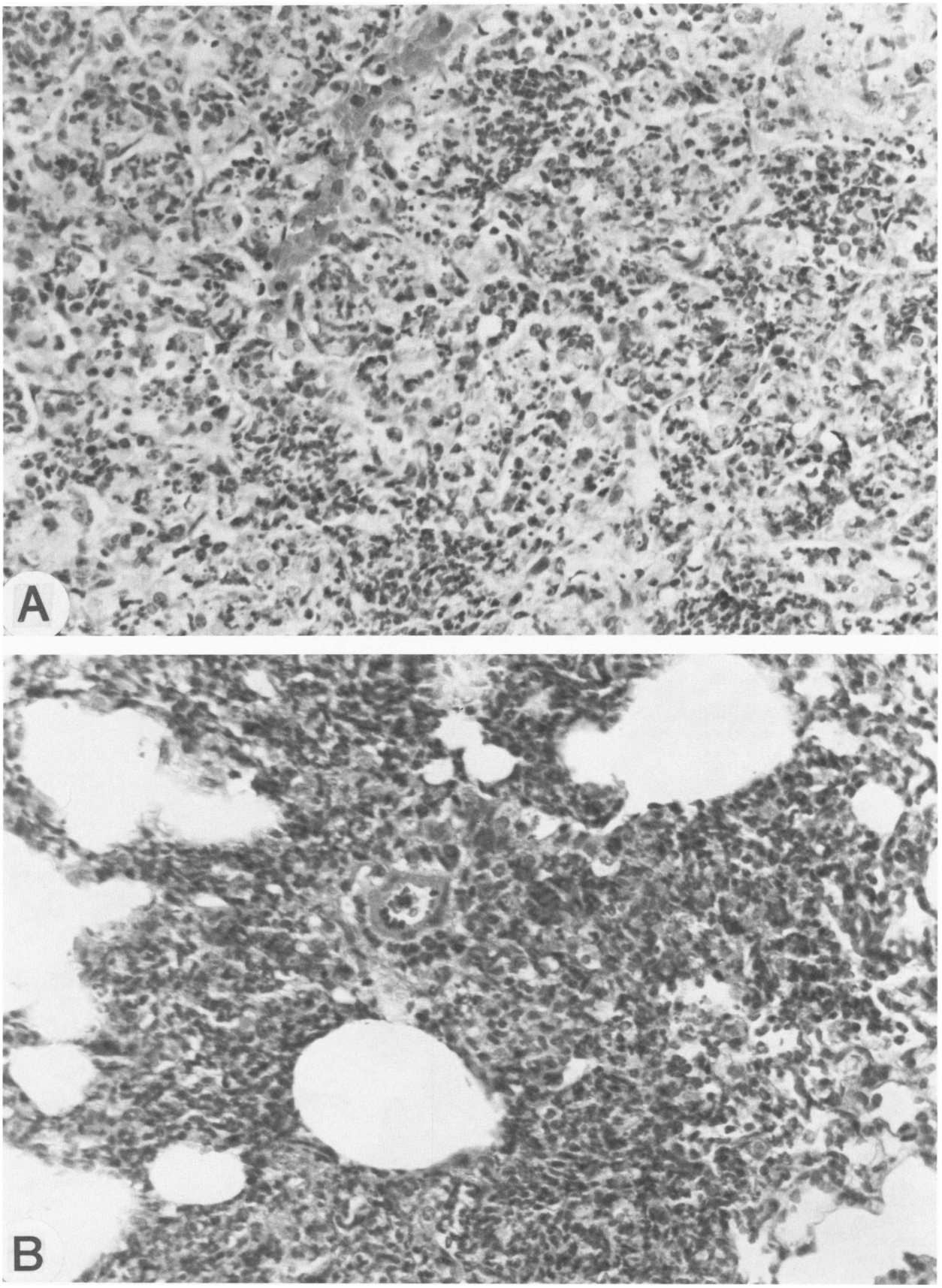
MIF Activity in BCG-Induced Granuloma Extracts

In our previous study, it was shown that the MIF activity in the granuloma extracts was associated with development of the lesion elicited by the intratracheal injection of sensitizing antigen-conjugated agarose beads into immunized guinea pigs and mice.[1,2] In the present study, significant MIF activity was found in the extracts prepared from C57BL/6J and BALB/c mice bearing granulomatous inflammation induced by intratracheal BCG challenge (Figure 2). MIF activity was detected in these high granuloma responder strains as early as 1 day after BCG challenge and persisted at high levels through day 7. Similar activity was seen in immunized and nonimmunized high granuloma responder mice given injections of BCG. Lung extracts prepared from immunized and nonimmunized high granuloma responder mice challenged with BCG immediately before sacrifice (day 0) showed no MIF activity, which demonstrated that BCG alone did not inhibit or enhance target cell migration. In contrast, low responder (CBA/J) mice showed less MIF activity in the extracts only on day 1 after BCG challenge. These results are consistent with observations by Neta and Salvin concerning murine strain variation of *in vivo* MIF release by intravenous injection with tuberculin into BCG-sensitized mice.[14]

Demonstration of IL 1 Activity in BCG-Induced Granuloma Extracts

To study the *in vivo* role of IL 1 in granulomatous inflammation, we assayed IL 1 activity in the granuloma extracts prepared from lungs of three different strains of mice. The results are shown in Figure 3. A significant amount of IL 1 activity was detected in granuloma extracts prepared from the high responder group (C57BL/6J and BALB/c mice). In both immunized and nonimmunized mice, peak activity was present 1 day after BCG challenge and rapidly declined thereafter to lower, still significant levels by day 7. In contrast to that in high responder mice, IL 1 activity in the extracts from low responder (CBA/J) mice given BCG is not only considerably less but also maintained for a shorter duration. In some experiments, high activity of IL 1 was detected as early as 6 hours after BCG challenge in immunized C57BL/6J mice (data not shown). These results are consistent with the histologic features of the lung (diffuse infiltration of mononuclear cells) and MIF activity in extracts from three different strains of mice challenged with BCG. In addition, BCG itself seemed to have no direct effect on this assay since extracts prepared from immunized and non-immunized mice injected with BCG immediately prior to sacrifice (day 0) showed no IL 1 activity. Interestingly, the highest level of IL 1 activity was observed in day 1 granuloma extracts prepared from immunized mice challenged with PPD-conjugated agarose beads (data not shown). In our previous study using other antigens (MBSA, egg albumin, keyhole limpet hemocyanin) conjugated beads

Figure 1—Histologic characteristics of the lung. **A**—Representative lesion seen in an immunized C57BL/6J mouse sacrificed 3 days after intratracheal challenge with viable BCG. Diffuse and intensive infiltration of mononuclear cells and few neutrophils. The infiltration was predominantly composed of macrophages, many showing abundant foamy cytoplasm, and lymphocytes. (H&E, ×250) **B**—Lung at 1 day after intratracheal challenge with BCG in an immunized BALB/c mouse. Severe, diffuse intraalveolar and interstitial inflammation. The infiltrate had a peribronchial distribution and was primarily composed of large macrophages, mature lymphocytes, and scattered neutrophils. (H&E, ×250) **C**—Section from the lung of an immunized CBA/J mouse given BCG 3 days previously. Only a very mild interstitial mononuclear cell infiltration can be seen. (H&E, ×400) **D** and **E**—Representative granuloma seen in immunized mice sacrificed 14 days (D:C57BL/6J) and 21 days (E:BALB/c) after BCG challenge. Histologic study of the lung showed randomly distributed, well-organized granulomas composed of epithelioid macrophages, multinucleated giant cells, and lymphocytes. (H&E, ×400) **F**—Pulmonary reaction in an immunized CBA/J mouse sacrificed 21 days after BCG injection. Rare and smaller epithelioid granulomas can be seen. (H&E, ×400)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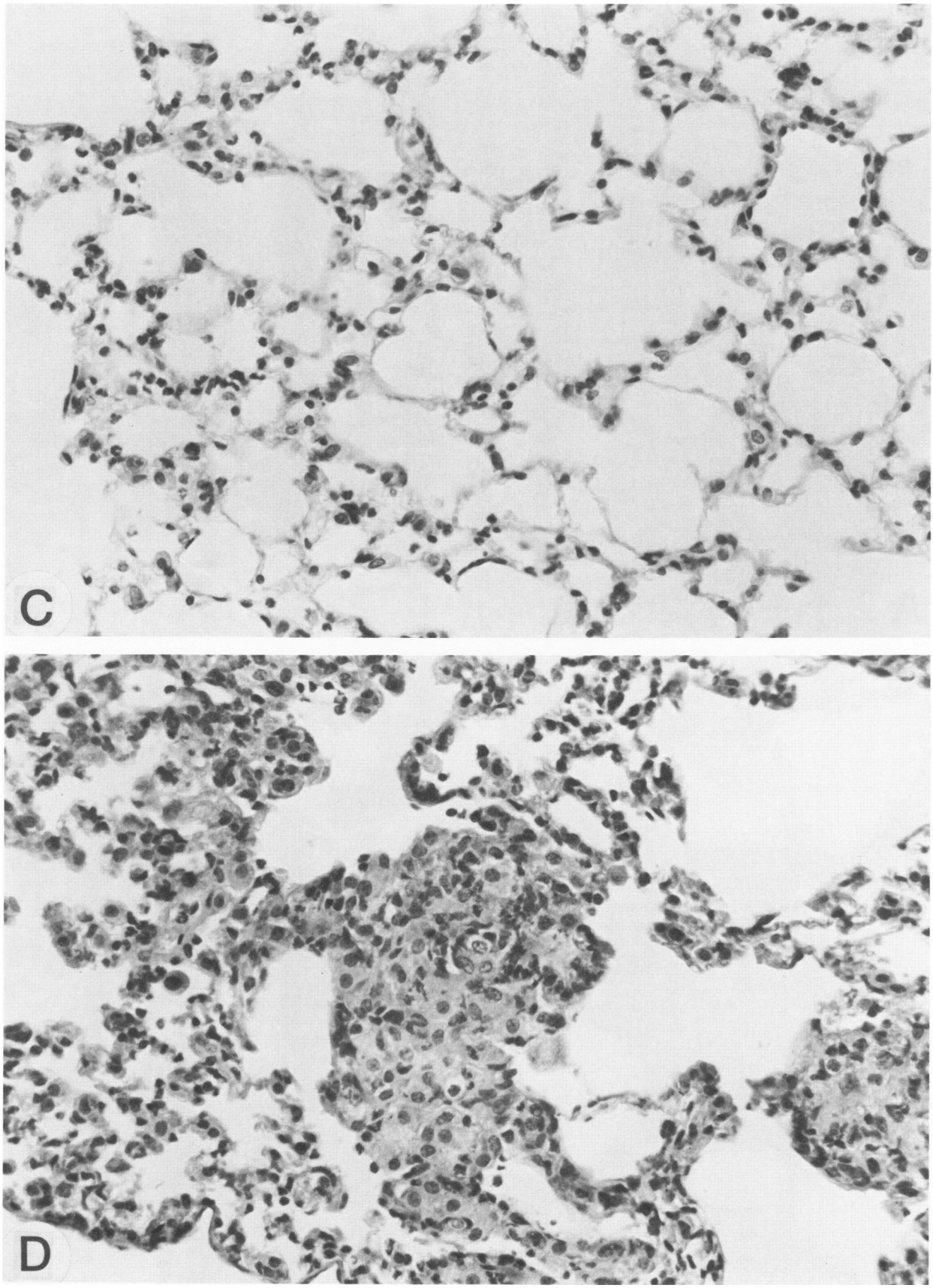
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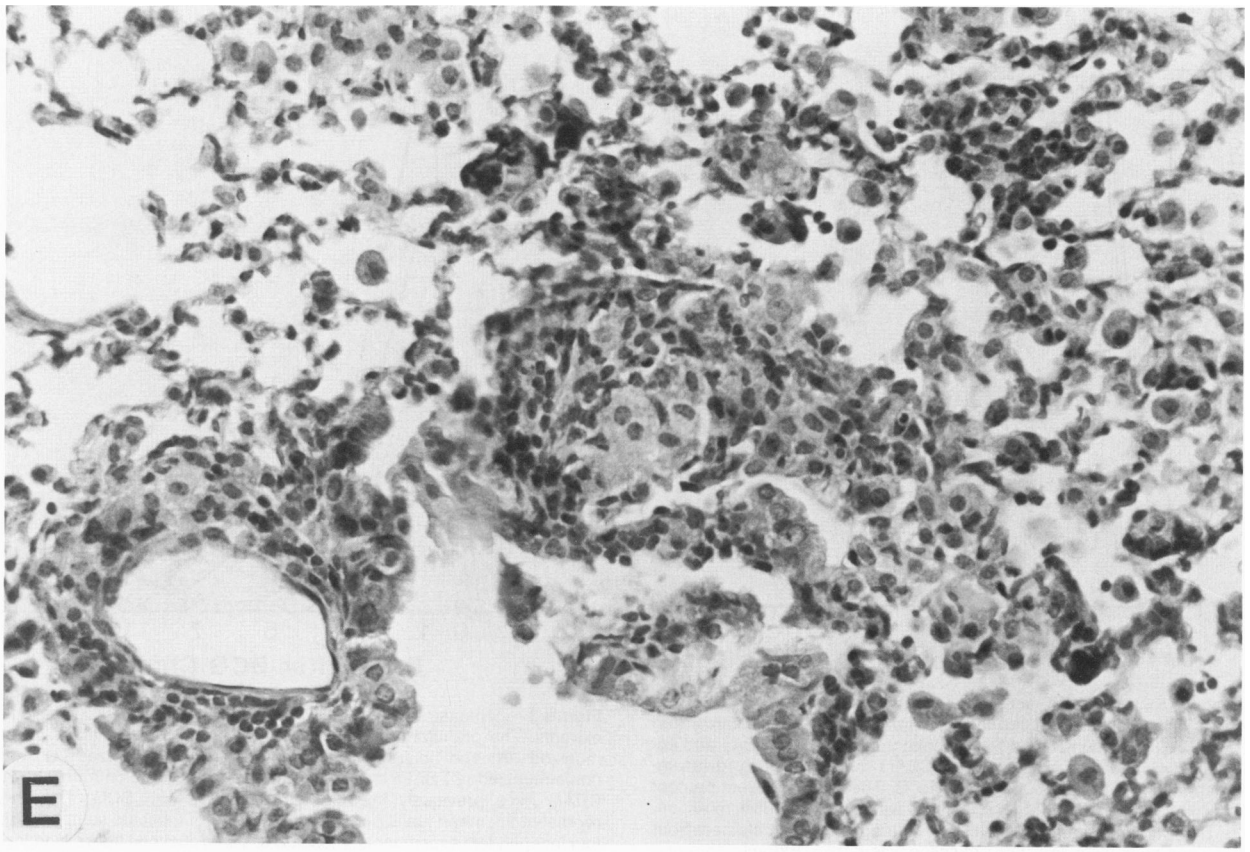
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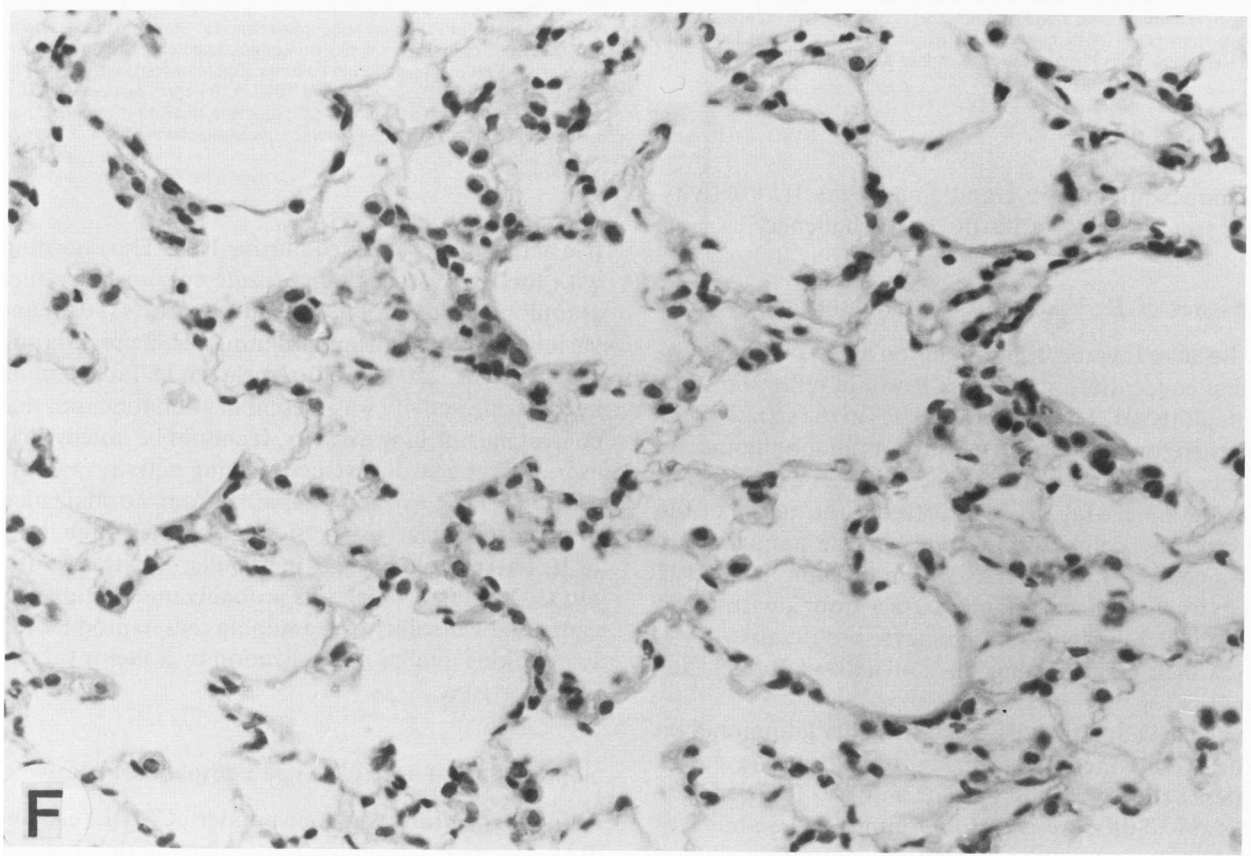
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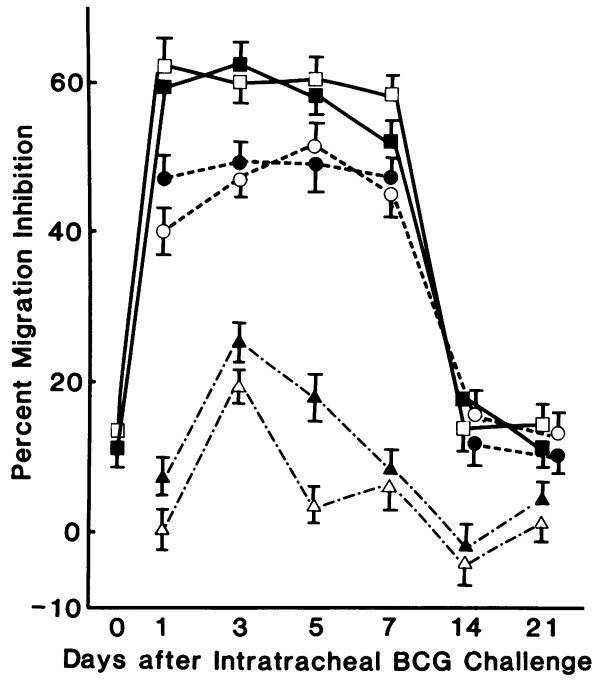

Figure 2—Kinetics of MIF activity in the granuloma extracts. Granuloma extracts were obtained from the lungs of immunized (■) and nonimmunized (□) C57BL/6J, immunized (●) and nonimmunized (○) BALB/c, and immunized (▲) and nonimmunized (△) CBA/J mice previously given intratracheal injections of 250 μg of live BCG. The data represent percent migration inhibition ± SEM as compared with those of RPMI 1640 control. The percent migration inhibition of lung extracts obtained from immunized mice given injections of saline was ⩽10%. Extracts from normal mice contained no significant activity (7 ± 3%) when compared with those from RPMI 1640 control. Results are combined from four replicate agarose MIF droplet assays from four separate experiments.

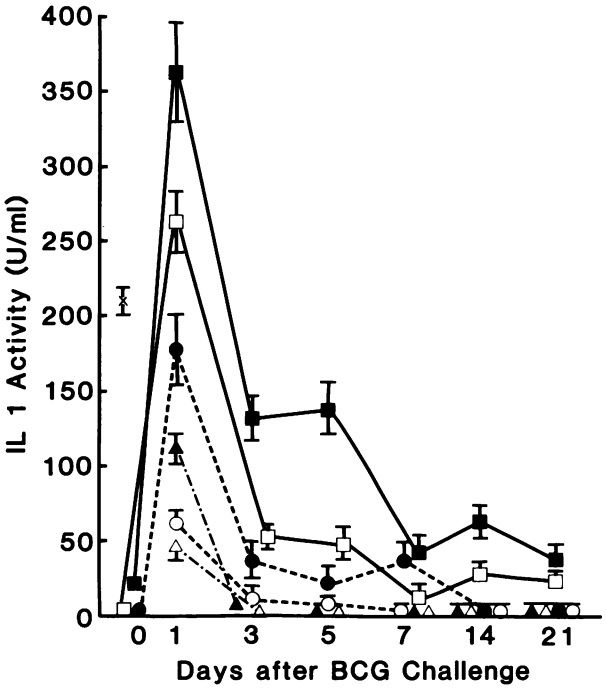

Figure 3—Demonstration of strain variation of IL 1 activity in granuloma extracts. The granuloma extracts were prepared from the lungs of immunized (■) and nonimmunized (□) C57BL/6J, immunized (●) and nonimmunized (○) BALB/c, and immunized (▲) and nonimmunized (△) CBA/J mice previously injected intratracheally with BCG. The data represent the mean (units per milliliter) ± SEM of triplicate samples from four independent experiments. Standard IL 1 was obtained from oil-induced BALB/c peritoneal exudate cells (5×10^5 cells/ml) incubated for 48 hours in the presence of 20 μg/ml lipopolysaccharide. Standard IL 1 showed 23060 ± 1060 cpm (X) at 1/4 dilution without detectable IL 2 activity. The levels of ^{3}H-TdR incorporation in cultures supplemented with only RPMI 1640 or PHA-P were 350 ± 26 and 1070 ± 93 cpm, respectively. IL 1 units were determined as described.[8] Extracts from all three strains of normal and immunized mice given intratracheal injections of saline showed no IL 1 activity.

to induce pulmonary granulomas, peak IL 1 activity was seen at 3 days after the bead challenge.[2]

Absence of IL 2 Activity in Granuloma Extracts

Because thymocyte proliferation assay with a suboptimal concentration of PHA-P would reflect both interleukins' (IL 1 and IL 2) activities in the samples,[15,16] we therefore examined IL 2 activity in granuloma extracts using strictly IL 2-dependent CTLL cells in a proliferative assay in order to clarify the nature of the thymocyte proliferation response in the standard IL 1 assay described above. As shown in Table 1, no IL 2 activity was detected in any extract from any strain of mice which had shown thymocyte proliferative activity, which indicates that the activity was, in fact, due to IL 1.

The next question asked was whether granuloma extracts contained neutralizing or interfering factor against the expression of IL 2 activity. To investigate this possibility, we therefore examined the ability of granuloma extracts to neutralize or inhibit proliferative activity of preformed murine IL 2. This was done with the IL 2 assay system, where cultures contained granuloma or control murine lung extracts (50 μl) and various dilutions of standard mouse IL 2 preparations (final volume, 250 μl/well). As shown in Table 2, mild neutralizing activity was present in granulomatous and control murine lung extracts. It should be noted, however, that the strongest neutralizing activity was observed in lung extracts prepared from normal mice. These results clearly suggest that extremely high levels of IL 1 activity are present in granuloma extracts without IL 2 activity, which was probably undetectable because of the inability of granuloma cells to produce this lymphokine and/or neutralization by a factor present in lung tissue.

Suppression of Delayed-Type Footpad Reactions

In high granuloma responder C57BL/6J and BALB/c mice with pulmonary inflammation induced

Table 1—Absence of IL 2 Activity in the Granuloma Extract

Granuloma extract source	Days after BCG injection	IL 2 activity (mean cpm ± SEM)* at dilution × 1/4	× 1/8
C57BL/6J			
Immunized	1	93 ± 6	86 ± 5
	3	57 ± 5	30 ± 4
	5	110 ± 7	71 ± 3
Nonimmunized	1	92 ± 3	81 ± 4
	3	78 ± 6	56 ± 4
	5	44 ± 5	107 ± 4
Normal		84 ± 8	92 ± 12
BALB/c			
Immunized	1	102 ± 8	81 ± 6
	3	116 ± 7	87 ± 5
Nonimmunized	1	86 ± 8	94 ± 12
	3	121 ± 16	99 ± 8
Normal		88 ± 6	113 ± 6
CBA/J			
Immunized	1	59 ± 9	ND†
	3	61 ± 8	ND
Nonimmunized	1	65 ± 7	ND
	3	63 ± 6	ND
Normal		65 ± 5	ND
Standard IL 2‡		26451 ± 326	28361 ± 987
Medium		197 ± 26	

* Mean counts per minute ± SEM of ^{3}H-TdR uptake in triplicate from four independent experiments.
† ND, not determined.
‡ BALB/c spleen cells (5 × 10^6 cells/ml) were incubated with 5 μg/ml Con A for 48 hours.

by BCG challenge, delayed-type footpad response to specific (BCG and PPD) and nonspecific (MBSA) antigens was significantly suppressed. No remarkable inhibition was found in BCG-challenged CBA/J mice. As shown in Figure 4, a marked diminution of footpad reactions was observed in the BCG-injected C57BL/6J and BALB/c mice from day 1 to day 7, while mild inhibition of antigen-induced delayed-type footpad responses was seen in immunized CBA/J mice challenged with BCG only on day 1. The mechanism of the abrogation of cutaneous delayed-type hypersensitivity reaction in these granuloma-bearing mice may be similar to that of anergy examined in the desensitization model of delayed-type hypersensitivity, as discussed later.[17]

Suppression of *in Vitro* Lymphocyte Proliferative Response in Granuloma-Bearing Mice

To determine the *in vivo* and *in vitro* correlation of the anergic state in granuloma-bearing mice, we examined the antigen- or mitogen-induced lymphocyte proliferation. Controls were a group of immunized syngeneic mice which received intratracheal saline challenge. Cells were obtained from lymph nodes of both granuloma-bearing mice and a control group of mice. High granuloma responder strains (C57BL/6J and BALB/c) showed marked suppression (50–80%) of PPD- and MBSA-elicited lymphocyte proliferation in immunized mice bearing BCG-induced pulmonary granulomatous inflammation (days 1–7). More moderate suppression (30–50%) with shorter duration (days 1–3) was seen when PHA-P was used to stimulate lymphocytes from these mice. These data were summarized in Figure 5A (C57BL/6J mice) and Figure 5B (BALB/c). In contrast, there was no significant difference between BCG-challenged and control immunized

Table 2—Nonspecific Neutralizing Activity of the Granuloma Extract Against Preformed Murine IL 2*

Granuloma extract† present during IL 2 assay	IL 2 activity‡ (cpm ± SEM) at various dilutions × 1/2	× 1/4	× 1/8	× 1/16
C57BL/6J				
Nonimmunized, BCG, day 1	28394 ± 578 (11.5)§	31132 ± 1317 (13.5)	28103 ± 776 (4.1)	14861 ± 542 (8.2)
Immunized, BCG, day 1	26409 ± 1913 (17.3)	29208 ± 917 (18.9)	22117 ± 516 (24.6)	13416 ± 257 (17.1)
Immunized, saline, day 1	27220 ± 913 (15.2)	32509 ± 708 (9.8)	23519 ± 951 (19.8)	13485 ± 627 (16.7)
Normal	24678 ± 2541 (23.1)	22949 ± 827 (36.3)	17896 ± 549 (39.0)	10789 ± 390 (33.4)
BALB/c				
Nonimmunized, BCG, day 1	28558 ± 597 (11.0)	32985 ± 1095 (8.4)	27469 ± 836 (6.3)	16297 ± 746 (–0.7)
Immunized, BCG, day 1	28612 ± 506 (10.8)	33929 ± 930 (5.7)	27485 ± 861 (6.2)	15676 ± 620 (3.1)
Immunized, saline, day 1	30561 ± 1048 (4.8)	36014 ± 352 (0)	29392 ± 449 (0)	16668 ± 623 (–3.0)
Normal	24338 ± 560 (24.2)	28805 ± 1028 (20.0)	22037 ± 383 (24.8)	11909 ± 74 (26.4)
CBA/J				
Nonimmunized, BCG, day 1	28382 ± 1485 (11.6)	30926 ± 775 (14.1)	25769 ± 1537 (12.1)	13565 ± 1291 (16.2)
Immunized, BCG, day 1	31190 ± 695 (2.8)	34154 ± 849 (5.1)	28152 ± 835 (4.0)	15679 ± 385 (3.1)
Immunized, saline, day 1	30055 ± 1171 (6.3)	34892 ± 1464 (3.1)	27634 ± 790 (5.7)	15935 ± 942 (1.5)
Normal	25494 ± 989 (20.6)	31859 ± 772 (11.5)	25718 ± 740 (12.3)	15574 ± 939 (3.8)
RPMI 1640	32073 ± 2380	35979 ± 1348	29300 ± 1570	16176 ± 1086

* IL 2 was derived from BALB/c spleen cells (5 × 10^6 cells/ml) incubated with 5 μg Con A for 48 hours.
† Fifty microliters of samples were added into IL 2 assay.
‡ Data represent mean counts per minute ± SEM of triplicate samples from three separate experiments, counts per minute of medium alone, was 265 ± 34.
§ Percent suppression against preformed IL 2 is in parentheses. Percent suppression indicates a decrease in preformed IL 2 activity in the presence of various samples when compared with the culture added RPMI 1640.

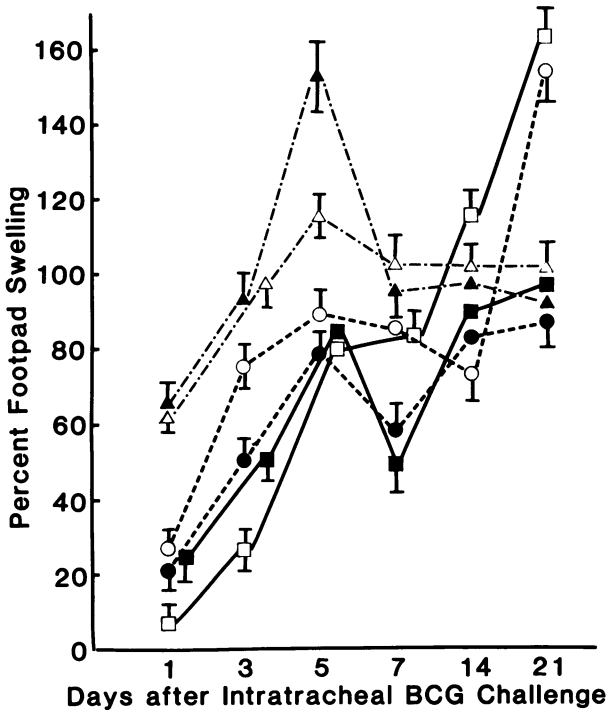

Figure 4—Kinetics of the footpad response to MBSA, PPD or BCG in different strains of mice. Sensitized mice were given intratracheal injection of BCG, and 24-hour footpad swelling in response to antigen challenge was evaluated at intervals 1–21 days later. C57BL/6J mice were tested with MBSA (□) or BCG (■); BALB/c mice were challenged with MBSA (○) or PPD (●); and CBA/J mice were injected with MBSA (△) or PPD (▲). Mean footpad swelling (millimeters) ± SEM in sensitized mice challenged intratracheally with saline was 0.48 ± 0.05 (MBSA) and 0.59 ± 0.07 (BCG) in C57BL/6J, 0.46 ± 0.06 (MBSA) and 0.27 ± 0.01 (PPD) in BALB/c, 0.29 ± 0.04 (MBSA) and 0.25 ± 0.03 (PPD) in CBA/J mice. The data represent the percent mean swelling ± SEM compiled from five different experiments.

CBA/J mice except very mild inhibition (20–30%) found in BCG-injected group only on day 1 (Figure 5C). The data indicate that the suppression of antigen- and PHA-P-induced *in vitro* lymphocyte proliferative response is temporally well associated with *in vivo* suppression of footpad delayed-type hypersensitivity reactions in granuloma-bearing mice.

Serum Antibody Response in Granuloma-Bearing Mice

The anergic state in the desensitization of delayed-type hypersensitivity by challenge with a large amount of antigen involves only the suppression of cell-mediated immunity. Thus, this type of immune suppression does not extend to the humoral immune response.[17,18] Therefore, we examined the serum IgG antibody response in BCG-induced, granuloma-bearing mice using a solid phase ^{125}I-protein A radioimmunoassay. As seen in Figure 6, anti-MBSA-specific serum IgG antibody production was detected approximately 2 weeks after immunization, then reached its peak, and plateau 2 weeks later. Mild inhibition of antibody production was observed in mice bearing BCG-induced pulmonary granulomatous inflammation during days 3–7. This is in sharp contrast to the marked suppression of *in vivo* and *in vitro* manifestations of cell-mediated immune responses seen in these animals.

Discussion

The data reported in this paper indicate that murine strain variation in granuloma formation responding to intratracheal challenge of viable BCG is clearly correlated with local production and/or release of soluble mediators such as MIF and IL 1 and the anergic state (selective suppression of *in vivo* and *in vitro* manifestations of cell-mediated immunity) in the granuloma-bearing mice. Anergy in granuloma-bearing mice was observed when the animals were actively producing and releasing these mediators. Recent studies from our laboratory have demonstrated that MIF and IL 1 activities are closely correlated with the development of pulmonary granuloma formation elicited by the intratracheal challenge of antigen-conjugated agarose beads into sensitized mice.[2] Interestingly, we have found the anergic state in such mice was associated with impaired production of IL 2 when lymph node cells were stimulated by antigen.[3] From these results, it is most likely that mechanism of granuloma formation and the associated anergy in mice injected with viable BCG may be similar to that in the sensitized animals injected with antigen-coated agarose beads.

Granuloma formation is the expression of a series of complex inflammatory event. Evidence has accumulated in recent years indicating that lymphokines may play an important role in the initiation and maintenance of granuloma formation.[1,2,19–21] Furthermore, it has been found that genetic factor(s) controls murine resistance[22–24] and granuloma formation[10,13] to BCG. The genetically resistant host is able to control the net bacterial growth without raising a significant immune response. The genetically susceptible host, on the other hand, responds to the increasing bacterial load by the prompt development of a specific immune response which is expressed by, for example, granuloma formation.[22,23,25] Based on these observations, the present study employed C57BL/6J and BALB/c mice as high granuloma producers (BCG-susceptible strain) and CBA/J mice as a low granuloma producers (BCG-resistant strain) to investigate immune responses and mediators which are responsible for granuloma formation. A significant amount of MIF and IL 1 activities was found in the granuloma extracts from the genetically susceptible mice (high granuloma producer),

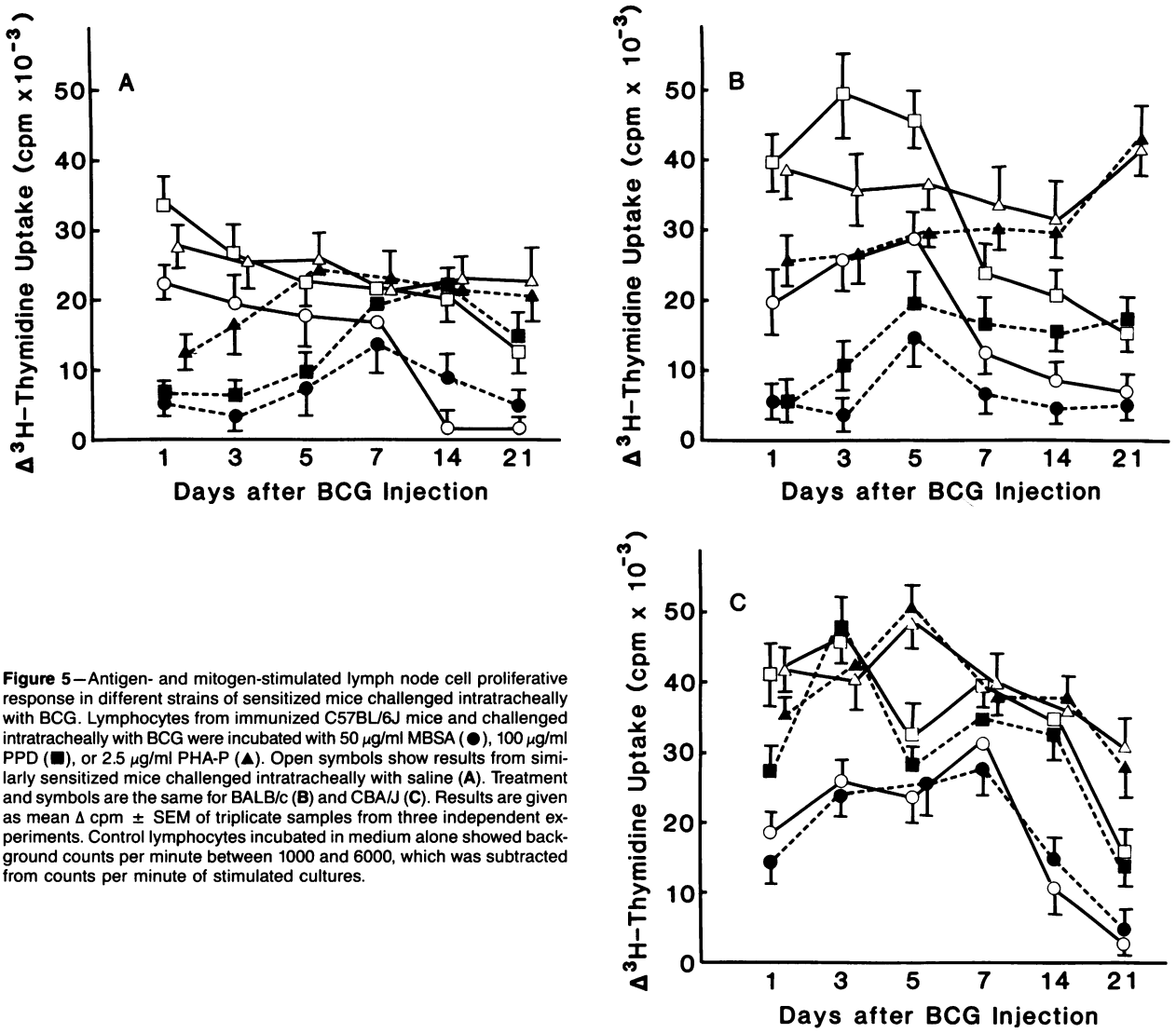

Figure 5—Antigen- and mitogen-stimulated lymph node cell proliferative response in different strains of sensitized mice challenged intratracheally with BCG. Lymphocytes from immunized C57BL/6J mice and challenged intratracheally with BCG were incubated with 50 μg/ml MBSA (●), 100 μg/ml PPD (■), or 2.5 μg/ml PHA-P (▲). Open symbols show results from similarly sensitized mice challenged intratracheally with saline (**A**). Treatment and symbols are the same for BALB/c (**B**) and CBA/J (**C**). Results are given as mean Δ cpm ± SEM of triplicate samples from three independent experiments. Control lymphocytes incubated in medium alone showed background counts per minute between 1000 and 6000, which was subtracted from counts per minute of stimulated cultures.

whereas a very small amount of these activities was detected in the genetically resistant mice (low granuloma producers). Moreover, these mediators in the lesions were detected as early as 6 hours after the intratracheal BCG challenge, which were associated with inflammatory cell infiltration in high granuloma producer strain of mice. These results clearly indicate that a product(s) of the gene regulating granuloma formation is expressed in the process of generating factors from activated macrophages (IL 1) and T-lymphocytes (MIF), which influences an early phase of host defense mechanism against BCG infection. Thus, these mediators may be closely related to granuloma formation. The activated T-lymphocytes in turn can stimulate macrophages to produce IL 1 either through direct cell contact[26] or through lymphokines.[27] It was shown that BCG possessed not only direct effects on macrophages to produce IL 1 but also adjuvant or indirect effects by activated T cells and/or lymphokines.[28,29] Alternatively, highly activated macrophages secreted less IL 1 by an increased content of inhibitors of RNA or protein synthesis.[30] Taken together with the present study, insufficient release and/or production of IL 1 and MIF in the CBA/J mice (BCG-resistant strain) may be responsible for low macrophage-T-lymphocyte response to BCG and for unremarkable granuloma formation.

Our results suggest that the anergy observed during the development of granulomatous inflammation in high-responder mice may involve a similar genetically determined mechanism. Thus, such gene products are probably expressed on macrophages because the BCG-induced anergy both *in vitro* and *in vivo* is caused by adherent Thy-1⁻, lg⁻ spleen cells in mice.[31,32] They may regulate not only granuloma formation but also devel-

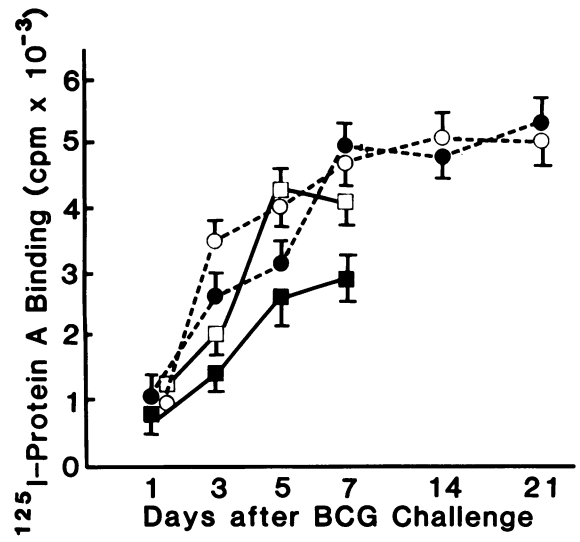

Figure 6—Detection of MBSA-specific serum IgG antibody in sensitized mice with BCG-induced granulomatous inflammation using the ^{125}I-protein A binding assay. Sera were obtained from MBSA-immunized C57BL/6J mice challenged intratracheally with BCG (■) or saline (□) and immunized BALB/c mice injected intratracheally with BCG (●) or saline (○). The values represent the mean counts per minute ± SEM of triplicate samples from two different experiments. Normal sera controls from C57BL/6J and BALB/c mice showed 740 ± 80 and 910 ± 60 cpm, respectively.

opment of anergy, and these two phenomena may be closely associated in a cause-and-effect relationship. Our recent study on desensitization as an interesting model of anergy has shown that the desensitization of delayed-type hypersensitivity was in part due to lymphokine-dependent mechanisms.[17] Thus, a large amount of circulating lymphokines generated and released at the initial stage of desensitization may be responsible for the general suppression of hypersensitivity reactions. In a subsequent step, monocytes and/or macrophages activated by these lymphokines release suppressive factor(s), including prostaglandin E_1 and E_2, which inhibit both the production and activities of lymphokines. Lymphokines are also suggested as being capable of directly inducing suppressor cells for lymphokine production, thus exerting a feedback inhibition.[17] In fact, the mechanism associated with anergy was also present in the murine granuloma model induced by intratracheal challenge of antigen-conjugated agarose beads. It was also suggested that the impaired antigen-elicited IL 2 production might be contributing to *in vivo* and *in vitro* manifestations of anergy.[3] Anergy observed in mice with BCG-induced granulomas is probably due to a similar mechanism because the temporal profile of MIF activity in the extract is closely related to a lack of manifestations of cell-mediated immunity. The results support the contention that the impaired T-cell proliferation is responsible for deficit IL 2 production in experimental mycobacterium infection.[33,34]

The link between IL 1 and IL 2 is an essential element in the cellular and humoral immune processes that are mediated by T cells[16,35,36] However, little is known about the role and interaction of IL 1 and IL 2 *in vivo*. Clinically, studies on patients with active pulmonary sarcoidosis have shown that the spontaneous release of IL 2 by the T cells in bronchoalveolar lavage fluid may have a central role in increasing the numbers of lung T cells.[37,38] Lung T cells are activated and release lymphokines such as monocyte chemotactic factor and MIF,[39] which are known to affect major cellular components within the lesion. The present results of MIF and IL 1 activities in the granulomatous lesions from high responder mice suggest that the activation of macrophages and T lymphocytes plays an important role in granuloma formation. In contrast, IL 2 was not detectable in the extracts. Instead, there was found the nonspecific lung factor to neutralize the preformed mouse IL 2. These results are in agreement with those in our previous study on granulomas induced by antigen-coated beads[2] and the fact that no IL 2 activity was found in the bronchoalveolar lavage fluid from patients with active pulmonary sarcoidosis,[37] although it was detectable in culture supernatant of lung T cells from the patients.[37,38] There are several possibilities to explain such an apparently conflicting phenomenon: 1) lack of IL 2 production in lung, 2) the presence of neutralizing or interfering factor(s) in the lavage fluid or lung tissue, or 3) absorption of IL 2 by antigen-activated T cells in the lesion.[40] Some of these can be definitely tested in future. In any event, macrophages are the most predominant cells in granulomatous lesions. Therefore, their lesions could evolve in the absence of detectable IL 2 if sufficient lymphokines relevant to macrophages such as MIF and macrophage-activating factor are available in the lesions. As we pointed out in the previous paper, the dissociation between IL 1 and IL 2 activities in the lesions may represent a general phenomenon in granulomatous disease with anergy.[2,41]

In conclusion, the present observations indicate that the interleukins and lymphokines play an important role not only in the BCG-induced granuloma formation but in the associated anergy, which suggests that this process is under genetic control.

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