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Enhanced Recovery of Murine Alveolar Macrophages: Morphological and Functional Characteristics following Intravenous Injection of Heat-Killed *Mycobacterium bovis* BCG

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The kinetics of induction of the bronchoalveolar cell population (i.e., alveolar macrophages [AM], lymphocytes, and polymorphonuclear leukocytes) was studied in mice inoculated intravenously with heat-killed *Mycobacterium bovis* BCG. Injection of BCG at 100 and 500 μg but not at 10 μg per mouse resulted in an increase in the total number of bronchoalveolar cells (threefold) and in the number of AM (sixfold) recovered by bronchoalveolar lavage in a time-dependent manner, as compared with control mice. A significant increase in the number of lymphocytes was also observed between days 2 and 4 after injection, but this number returned to normal levels by day 8, whereas the number of polymorphonuclear leukocytes was not significantly altered. AM were characteristically phagocytic and stained positively for nonspecific esterase. AM recruited in response to BCG injection were activated, as indicated by elevated levels of acid phosphatase activity and decreased levels of membrane 5′-nucleotidase activity. However, both resident and BCG-induced AM suppressed the in vitro plaque-forming cell response of sheep erythrocyte-primed mice to the same extent. These results indicate that injection of heat-killed BCG induced increased numbers of activated AM, which appeared to be functionally similar to resident AM in their ability to phagocytize and modulate in vitro immune responses.

Interest in pulmonary alveolar macrophages (AM) as a key element in the defense of the lung began to flourish over the past 10 years as a consequence of the development of bronchoalveolar lavage techniques which permitted the recovery of AM first from animals (16) and later from humans (21). In recent years, a great deal of attention has been focused on attempts to define the role of these cells in immune responses. The results of numerous studies have revealed a complex and frequently conflicting picture (8). Investigation of the immunological function(s) of AM in small laboratory animals has been greatly hampered by the relatively low number of cells recoverable by bronchoalveolar lavage techniques (15). Several attempts have been made to improve the cell yield, beginning with a careful study of the factors which influence the lavage procedure itself (4). Intravenous administration of heat-killed *Mycobacterium bovis* BCG emulsified in oil-water has resulted in an increased recovery of AM from mice (2) and rabbits (17). It has also been shown that lidocaine (Xylocaine; Astra Pharmaceutical Products, Inc., Westboro, Mass.; lignocaine), a local anesthetic, rapidly and reversibly detaches a variety of adherent cells from tissue culture plates (20). When incorporated into lavage fluid, lidocaine subsequently increased bronchoalveolar cell (BAC) recovery, with no reported effects on the morphology or functional properties of AM recovered from rats, mice, or guinea pigs (10). In another study, however, it was demonstrated that exposure to lidocaine had profound effects on the function of human immunocompetent cells (1). Other investigators have used chelating agents (EDTA) or enzymes (pronase) to increase the recovery of AM (3), but these agents were shown to induce structural and biological changes in the macrophages.

The present study was carried out to obtain relatively large numbers of functionally unaltered AM from mice for in vitro studies of the immunoregulatory functions of AM. We report that intravenous injection of sonified heat-killed BCG markedly increased the recovery of AM from BALB/c mice and that the addition of lidocaine to the lavage fluid significantly augmented the cell yield. We also describe the kinetics of induction of the total BAC population and characterize adherent AM with respect to their phagocytic, cytochemical, enzymatic, and immunomodulating properties.

MATERIALS AND METHODS

Mice. Virus-free male BALB/c mice, 5 to 6 weeks old, were obtained from Harlan Sprague-Dawley, Inc., Walkersville, Md. They were maintained at the Georgetown University Animal Care Facility in accordance with National Institutes of Health guidelines. The mice were provided with food and water ad libitum.

Administration of BCG. Lyophilized heat-killed BCG was purchased from Quentin Myrvik, Bowman-Gray School of Medicine, Winston-Salem, N.C. BCG was homogenized in phosphate-buffered saline (PBS) by ultrasonic-probe sonication (Heat Systems Ultrasonics Inc., Plainview, N.Y.) for three 15-s intervals at 50 cycles/s. Seven- to ten-week-old mice were inoculated in the tail vein with 0.5 ml of the appropriate concentration of BCG homogenate. Control mice were injected with PBS, unless indicated otherwise.

Bronchoalveolar lavage. BACs (lymphocytes, AM, and polymorphonuclear leukocytes [PMN]) were isolated at different times from the lungs of mice inoculated with BCG by lavage as described previously (15). Lung lavage was carried out with warm (37°C) Ca^{2+}-, Mg^{2+}-free PBS containing 17 mM lidocaine hydrochloride. The lungs of each mouse were lavaged with a total of 5 ml of fluid, with only 0.5 ml being instilled at a time. BACs were placed on ice immediately, washed three times with Ca^{2+}-, Mg^{2+}-free PBS, and counted in a hemacytometer, and viability was determined by trypan blue dye exclusion.

Characterization of BAC. (i) Cytochemical staining. Cytocentrifuge preparations of BACs (10^6 cells per ml) were dried

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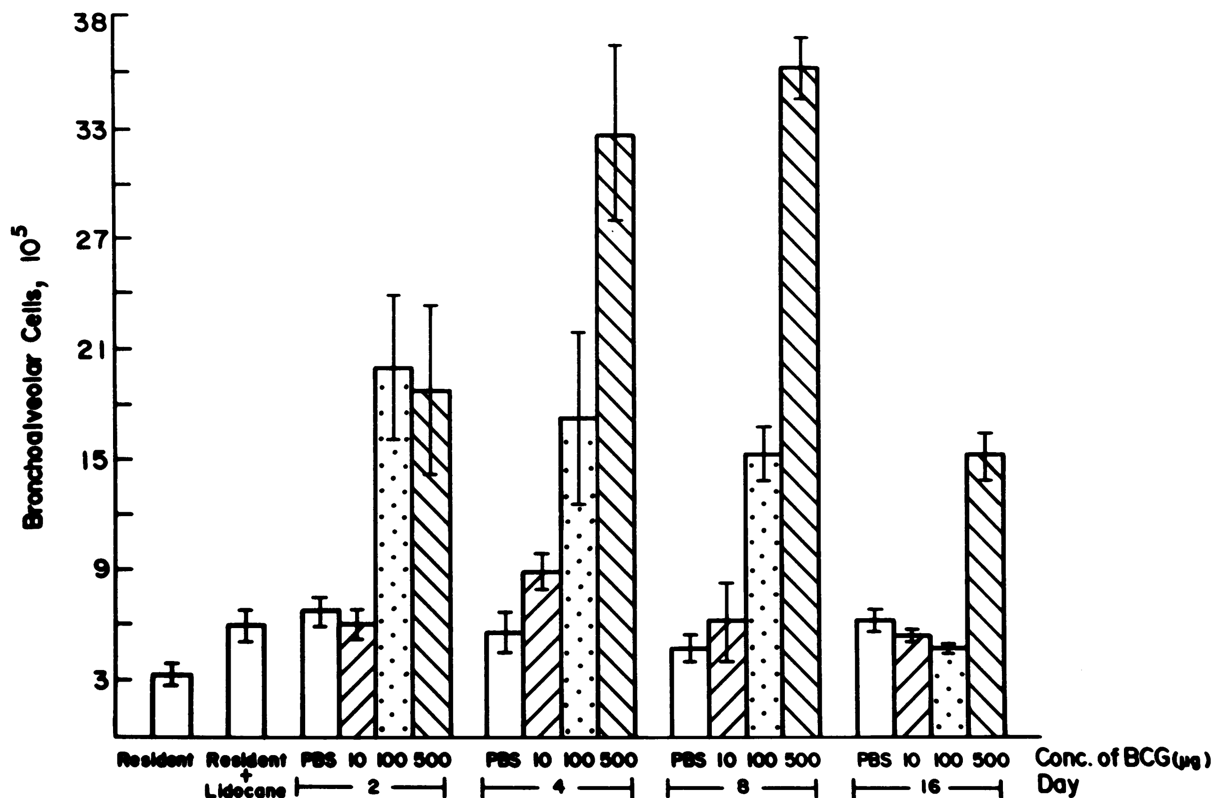

FIG. 1. Kinetics of recruitment of BACs in mice inoculated intravenously with different concentrations (Conc.) of BCG. Lavage fluid used for all but the resident group contained 17 mM lidocaine. Control groups were untreated (resident) or inoculated with PBS. Values are presented as the mean ± standard error of the mean for three to four experiments.

in air, fixed, and stained with Wright stain (Diff-Quick; American Scientific Products, McGaw Park, Ill.). Differential cell counts for AM, lymphocytes, and PMN were made on a total of 100 randomly chosen cells. Nonspecific esterase was demonstrated cytochemically with α-naphthyl acetate (26).

(ii) Immune phagocytosis. BACs were suspended in RPMI-1640 (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum (GIBCO) at 10^6 cells per ml. Samples (1-ml) were allowed to adhere to glass cover slips placed in 35-mm petri dishes (no. 1008; Becton Dickinson Labware, Oxnard, Calif.) at 37°C in a humidified incubator containing 5% CO_2 in air for 1 to 2 h. Nonadherent cells were removed by vigorous washing with warm medium. A 1-ml amount of a suspension of 5% sheep erythrocytes (SRBC) coated with a subagglutinating titer of rabbit anti-SRBC was added to the adherent cells and incubated at 37°C for 1 h. Nonphagocytized SRBC were removed by washing, and the cover slips were stained with Diff-Quick. A total of 100 macrophages were counted at random, and the percentage of cells phagocytizing SRBC was quantitated on the basis of whether they engulfed more than five or less than five antibody-coated SRBC.

(iii) AM-mediated suppression of the in vitro plaque-forming cell response. Seven- to ten-week-old BALB/c mice were primed with 0.2 ml of 10% SRBC in PBS injected intraperitoneally. Four days later, 10^7 immunized spleen cells were incubated with 2×10^6 SRBC with or without different numbers of AM in a total volume of 1 ml of RPMI-1640 medium containing 10% fetal calf serum in a 24-well tissue culture plate (no. 3424; Costar, Cambridge, Mass.) at 37°C in a humidified incubator containing 5% CO_2 in air for 3 to 4 days. The cells were harvested, and the number of cells secreting anti-SRBC antibody was measured in a hemolytic plaque assay (19).

(iv) Enzymatic studies. BACs (3 ml) suspended in PBS at 2×10^6 cells per ml were placed in 35-mm petri dishes and incubated at 37°C for 2 h. Nonadherent cells were removed by washing with warm PBS. AM were solubilized in 0.5 ml of 0.05% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.). The cell lysate was centrifuged at 600 × g for 10 min at 0 to 4°C to remove precipitated DNA. The supernatant was used for acid phosphatase, 5′-nucleotidase, and protein determinations.

Acid phosphatase was assayed in the lysate by measuring the amount of p-nitrophenol liberated from p-nitrophenyl phosphate (Sigma) in 50 mM citrate buffer at pH 5.0 (5, 24). The assay mixture containing 0.5 ml of 5.5 mM p-nitrophenyl phosphate and 0.1 ml of lysate was incubated in a water bath at 24°C for 1 h. The reaction was stopped by the addition of 1 ml of ice-cold 0.1 N NaOH containing 0.001 M EDTA. The A_{405} was measured in a model DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Acid phosphatase from potatoes (no. P3752; Sigma) was used as a positive control. An extinction coefficient (slope of the regression line of the plot of p-nitrophenol standard in nanomoles per milliliter versus citrate buffer blank) was determined. One unit of enzyme activity was defined as the liberation of 1 nmol of p-nitrophenol per mg of protein per min at 24°C (18).

Protein was determined by a modification of the procedure of Lowry et al. (14) by substituting sodium citrate for sodium potassium tartarate.

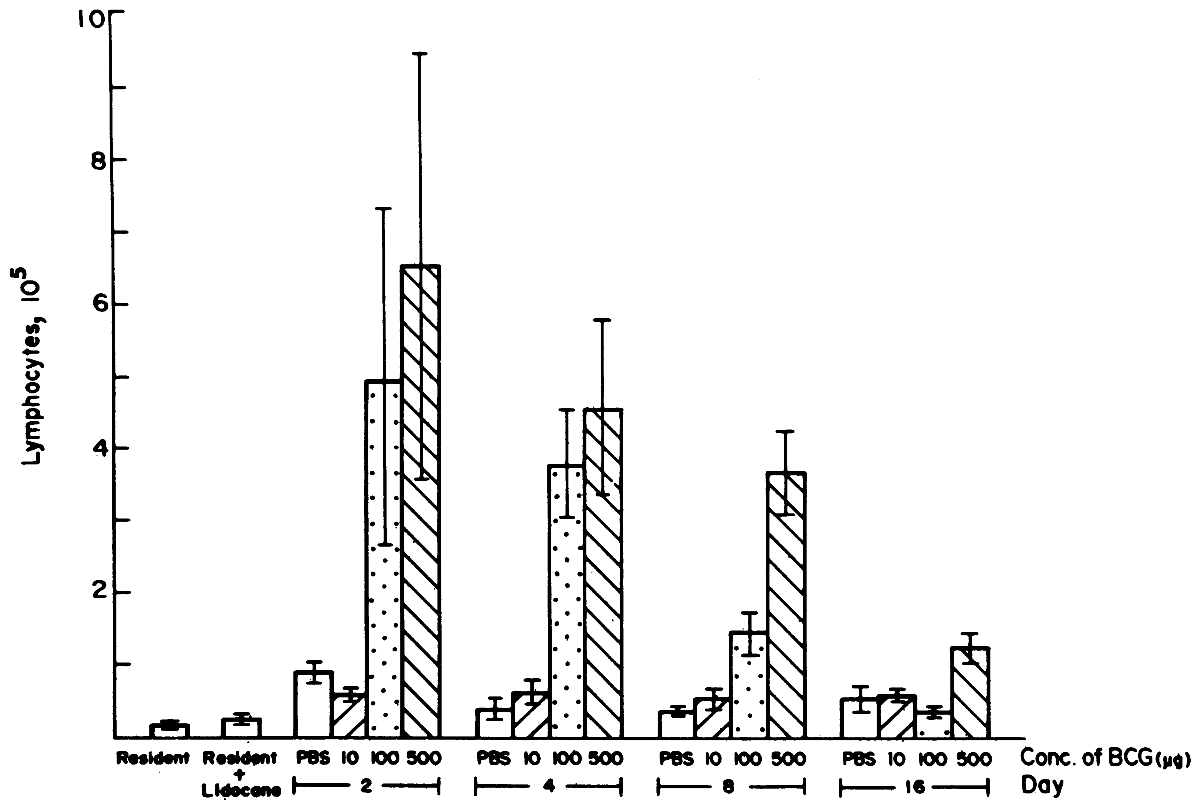

FIG. 2. Number of lymphocytes (mean ± standard error of the mean) in lavage fluid as determined by differential counting of Wright-stained smears or cytocentrifuge preparations of BACs. See the legend to Fig. 1 for more information.

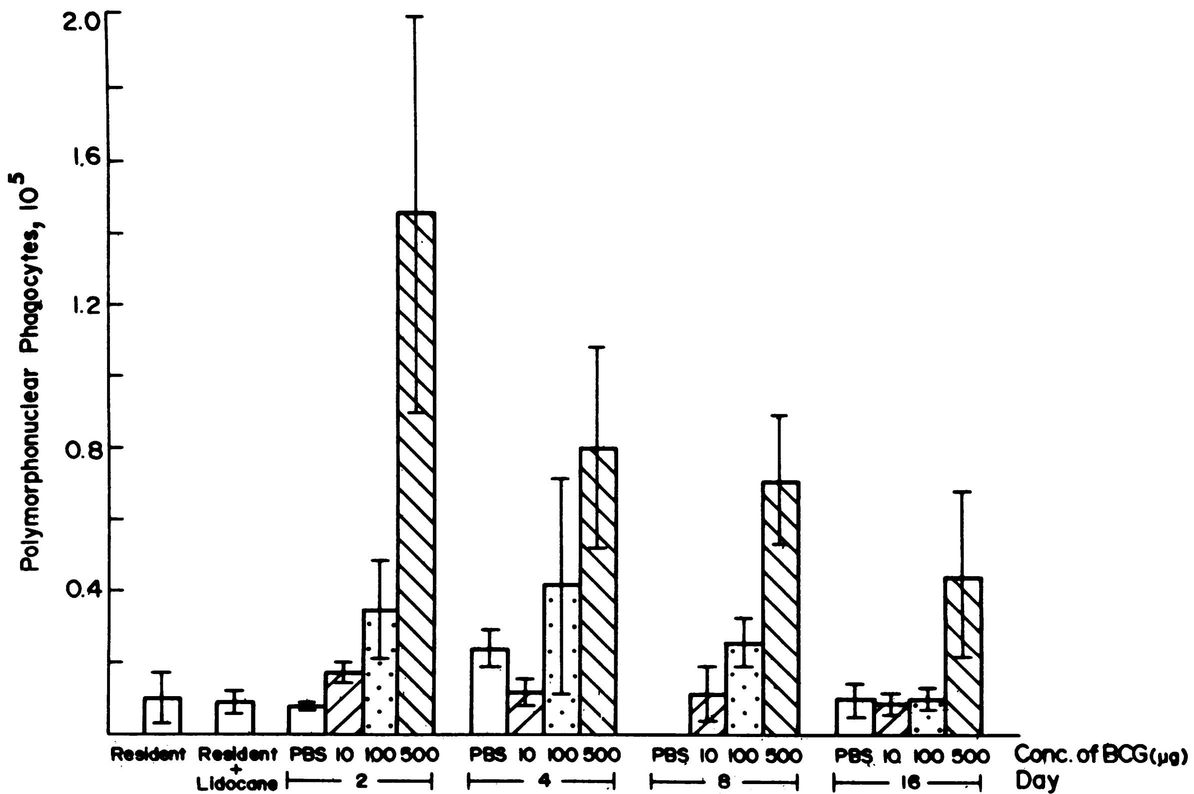

FIG. 3. Number of PMN in the BAC population as determined by differential counting of Wright-stained smears or cytocentrifuge preparations of BACs. Values represent the mean ± standard error of the mean for three to four determinations. See the legend to Fig. 1 for more information.

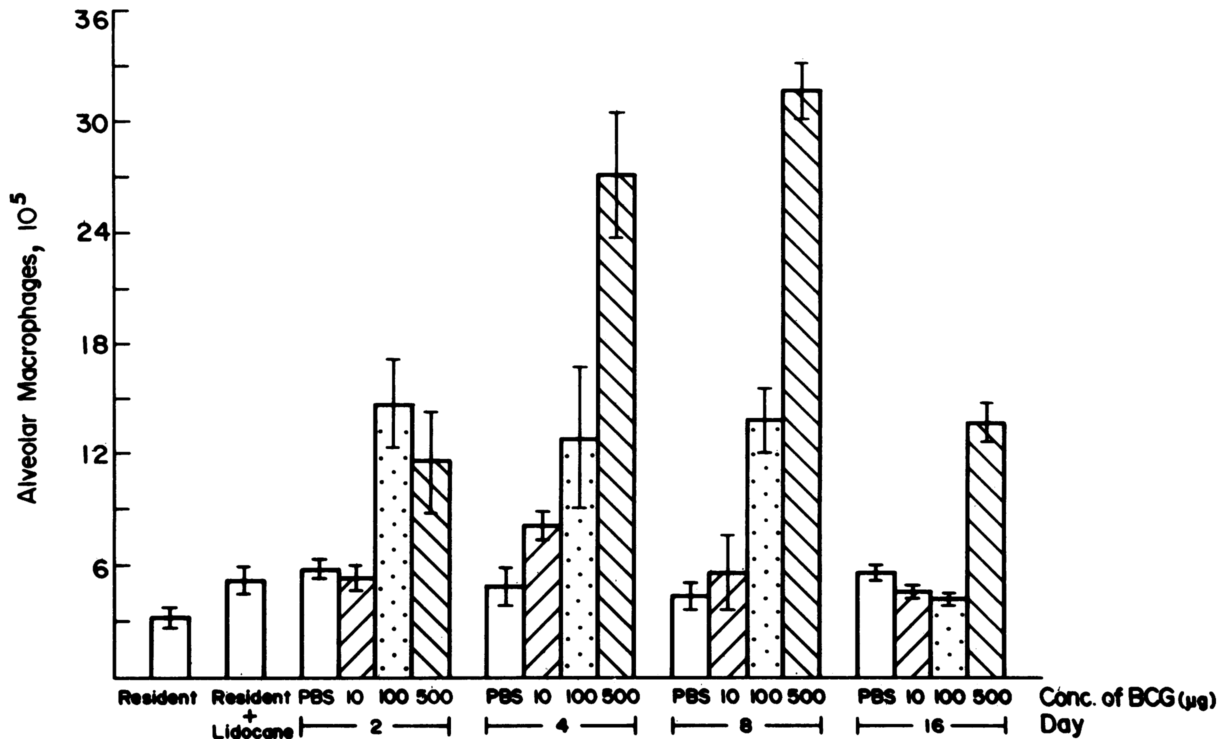

FIG. 4. Number of AM in the BAC population as determined by differential cell counting of Wright-stained smears or cytocentrifuge preparations of BACs. Values represent the mean ± standard error of the mean for three to four determinations. See the legend to Fig. 1 for more information.

Datum analysis. Pairwise t tests of differences between the means of different treatment groups were performed by using the Statistical Analysis System (SAS Institute, Cary, N.C.).

RESULTS

Kinetics of induction of BACs after intravenous administration of heat-killed BCG. The recovery of BACs showed a time- and concentration-dependent increase in the total number after BCG injection. Two days after injection of 100 or 500 μg of BCG, there was more than a threefold increase in the number of BACs, as compared with the number obtained from animals injected with either PBS or 10 μg of BCG ($P < 0.0001$). Mice injected with 500 μg of BCG showed the greatest increase in the total number of BACs, which peaked by day 8 and began to decline by day 16 (Fig. 1). Subsequent studies showed that the number of cells returns to the preinjection level by about day 25 (data not shown). Note that the incorporation of 17 mM lidocaine into the lavage fluid resulted in a twofold increase in the number of resident BACs obtained from unimmunized animals (Fig. 1). For this reason, in all subsequent studies the lavage fluid was supplemented with lidocaine. Also, the use of 10 ml instead of 5 ml of lavage fluid resulted in a further 20 to 30% increase in the number of cells recovered (data not shown).

Lymphocytes, which constitute 5 to 10% of resident BACs, also increased significantly in number after BCG injection. The increase in the number of lymphocytes corresponded to the increase in the total number of BACs recovered (Fig. 2). Hence, at 2 days, the percentage of lymphocytes recovered from the lungs of mice injected with 100 and 500 μg of BCG was 15 to 25% of the total BAC population, but this number returned to normal levels by day

TABLE 1. Functional and cytochemical characterization of pulmonary AM

Treatment	% Phagocytosed cells[a]		% Esterase-positive cells[b]
	<5 SRBC/cell	>5 SRBC/cell	
Day 0 (resident)	8	90	96
Day 2			
Control (PBS)	7	91	95
BCG (10 μg)	6	90	96
BCG (100 μg)	14	79	94
BCG (500 μg)	15	77	94
Day 4			
Control (PBS)	7	91	96
BCG (10 μg)	11	84	93
BCG (100 μg)	5	89	93
BCG (500 μg)	10	84	93
Day 8			
Control (PBS)	7	87	93
BCG (10 μg)	7	88	95
BCG (100 μg)	11	85	92
BCG (500 μg)	5	88	97
Day 16			
Control (PBS)	7	87	93
BCG (10 μg)	7	88	95
BCG (100 μg)	11	85	92
BCG (500 μg)	5	88	97

[a] Expressed as the percentage of AM which phagocytized less than or more than five antibody-coated SRBC per cell when 100 AM were counted at random. There were no significant differences between the groups, as determined by paired t-tests ($N = 9$).

[b] Nonspecific esterase was determined with α-naphthyl acetate as the substrate.

TABLE 2. Comparison of acid phosphatase activity in resident and activated AM and PM[a]

Treatment	Acid phosphatase activity (nmol/min per mg of protein) on day:			
	1	4	8	16
AM				
Control (PBS)	22,711 ± 135	25,690 ± 25	16,106 ± 52	27,978 ± 287
BCG (10 μg)	23,524 ± 38	31,582 ± 35	35,126 ± 122	30,407 ± 317
BCG (100 μg)	24,520 ± 151	49,774 ± 56	59,079 ± 192	31,128 ± 43
BCG (500 μg)	19,461 ± 137	53,042 ± 85	57,211 ± 109	46,784 ± 172
PM				
Control (PBS)	925 ± 47	1,523 ± 21	1,115 ± 65	611 ± 39
BCG (500 μg)	2,270 ± 52	6,118 ± 152	2,611 ± 182	1,533 ± 123

[a] Triton X-100 lysates of adherent resident and BCG-activated AM and PM were assayed for cytoplasmic acid phosphatase with *p*-nitrophenyl phosphate as the substrate.

8. The number of recoverable PMN did not appear to be changed significantly as a consequence of BCG injection (Fig. 3).

The number of AM obtained by lung lavage was also significantly increased in mice inoculated with 100 and 500 μg of BCG, in a pattern similar to that for BACs. Injection of 500 μg of BCG induced the greatest number of AM, more than a sixfold ($P < 0.0001$) increase by day 8, and this number remained elevated up to day 16 (Fig. 4). The number of AM recovered from the lungs of BCG-injected mice returned to normal levels by day 25 (data not shown).

Characterization of AM. Fc-dependent phagocytosis of immunoglobulin G-coated SRBC by adherent AM is shown in Table 1. The results are presented as percentages of phagocytic cells, that is, AM which engulfed less than or more than five SRBC per cell when 100 AM were counted at random. AM induced by injection of 100 or 500 μg of BCG tended to be slightly less phagocytic of more than 5 SRBC per cell than cells recovered from animals injected with PBS or 10 μg of BCG at day 2. However, when total phagocytosis (sum of the cells phagocytizing <5 and >5 SRBC) was computed, there was no difference between any of the groups. It should be pointed out that differences may be difficult to detect since the assays were performed under optimal conditions.

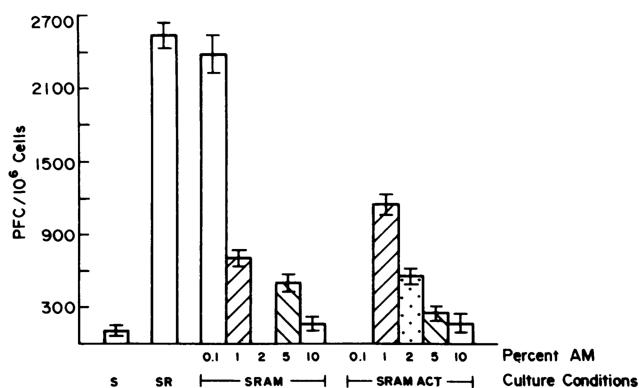

FIG. 5. Suppression of the in vitro plaque-forming cell (PFC) response of spleen cells from SRBC-primed mice by resident and activated AM. Spleen cells (10^7) from immunized animals were cultured alone (S) or cocultured with 2×10^6 SRBC (SR), with SRBC plus resident AM (SRAM), or with SRBC plus activated SRAM (SRAM ACT) to yield AM/lymphocyte ratios of 1:10 (10^6 AM), 1:20 (5×10^5 AM), 1:50 (2×10^5 AM), 1:100 (10^5 AM), or 1:1,000 (10^4 AM). Plaque-forming cell responses were measured after 3 days of culturing and are expressed as the number of plaque-forming cells per 10^6 cells ± standard error of the mean for 3 to 12 determinations.

Cytochemical characterization of adherent monolayers of AM showed that 92 to 97% of these cells were positive for nonspecific esterase, with no appreciable differences between different treatment groups (Table 1). It should be noted that the experimental groups contained significantly larger granular macrophages.

Enzymatic studies. Acid phosphatase, a cytoplasmic lysosomal hydrolase, is known to be elevated in activated macrophages. The effect of BCG injection on the activity of this enzyme was studied in AM recovered by lung lavage (Table 2). BCG at 500 and 100 μg significantly increased the levels of acid phosphatase activity in lavaged AM, as compared with AM recovered from mice inoculated with PBS or 10 μg of BCG ($P < 0.0001$). The enzyme activity peaked between days 4 and 8 and declined to normal levels by day 16. A comparison of acid phosphatase levels in AM with the activity of this enzyme in peritoneal macrophages (PM) indicated that resident AM had significantly higher levels of activity than did resident PM, an indication that AM may be more activated than PM under normal conditions. PM were also responsive to activation with 500 μg of BCG, which stimulated acid phosphatase activity up to sixfold by day 4; this activity declined to normal levels by day 16. In preliminary studies, resident AM had significantly lower levels of plasma membrane 5'-nucleotidase activity than resident PM ($P < 0.001$), whereas BCG-activated AM and PM lower activities than their nonstimulated counterparts (unpublished results).

AM-mediated suppression of the in vitro plaque-forming cell response. It was previously shown that the addition of AM at a concentration of 10% with respect to immunized splenocytes caused a greater than 90% suppression of the in vitro plaque forming cell response (19). The ability of normal (resident) and BCG-induced (500 μg) AM to mediate this suppression was therefore compared. Both resident and activated AM were equally suppressive in a concentration-dependent manner (Fig. 5). This indicates that for at least this parameter, resident and BCG-induced AM are biologically and functionally similar. In contrast, when tested in the same system, activated PM were more suppressive than resident PM (unpublished results).

DISCUSSION

The results described here clearly demonstrate that intravenous injection of a sonified homogenate of heat-killed BCG together with the inclusion of lidocaine in the lavage

fluid effectively increased the recovery of AM from the lungs of BALB/c mice. The recovery of both total BACs and AM was BCG dose and time dependent. The influx of macrophages, presumably from the circulation (2, 3), as has already been demonstrated to occur with PM (23) and Kupffer cells (7), attained an average value of 3.1×10^6 cells recoverable from the lungs of mice inoculated with 500 μg of BCG 8 days earlier. This represents an approximate sixfold increase over the normal steady state of 5.8×10^5 cells recovered from a mouse.

Lymphocyte contamination, which was as high as 31% of the total BAC population recovered from the lungs of mice inoculated with 500 μg of BCG 2 days earlier, returned to base-line levels by day 8. The contaminating lymphocytes could, however, be removed easily by allowing the BAC population to adhere at 37°C for 1 to 2 h, followed by removal of the nonadherent lymphocytes by gentle washing. This procedure always resulted in a 95 to 98% recovery of viable AM and eliminated the potential contribution of immunocompetent lymphoid cells, which could alter the interpretation of studies of immune function involving AM. In instances in which prior manipulation of the BAC population might be undesirable, it is recommended that lung lavage be carried out 8 days after BCG injection, when the number of recoverable lymphocytes is minimal. In our experience, there have been no detectable differences in results after the use of unfractionated BAC populations or lymphocyte-depleted AM populations. As has been observed previously (6), PMN constituted only a small proportion (2%) of the resident BAC population and were not significantly changed as a result of BCG stimulation (Fig. 3). It should be noted that lavage fluid recovered from the lungs of mice inoculated with either PBS or BCG exhibited marked contamination with erythrocytes 2 days later, but this was not observed if the cells were collected on day 4 after injection. The presence of erythrocytes in the lavage fluid may be associated with the trauma caused by the infusion of 0.5 ml of fluid into the lungs.

The incorporation of lidocaine into the lavage fluid was shown to result in an almost twofold increase in the number of resident AM recovered from the lungs of unstimulated animals. Based on this observation, we also added lidocaine to the fluid used for lavaging the lungs of BCG-immunized mice in an attempt to further augment the yield of AM. In a previous study, the addition of up to 12 mM lidocaine to lavage fluid was shown to increase the number of AM recovered from the lungs of mice, rats, and guinea pigs by up to 10-fold, as compared with cells recovered in the absence of this material (10). Why the yield obtained in the previous study was so much greater remains to be defined. There are several reports in which it has been demonstrated that both prolonged and short-term exposure to lidocaine results in alterations in the structure and function of immunocompetent cells. Reversible inhibition of hexose monophosphate shunt activity in human AM has been observed after short-term exposure to lidocaine (9). Decreases in cell viability (25), phagocytic capacity (11), and mitogen responsiveness (1) have been observed after lidocaine exposure. In the present study, lidocaine was added at a concentration of 17 mM without any demonstrable deleterious effects on cell viability (98% viable) or function.

As expected, inoculation of mice with BCG resulted in a dose- and time-dependent increase in the state of activation of adherent AM, as indicated by elevated levels of acid phosphatase activity (Table 2). Others have shown that the stimulation of mouse PM with BCG and other inflammatory agents both in vivo and in vitro resulted in elevated levels of acid phosphatase activity (12, 22). Similar results were obtained in this study (Table 2) when enzyme activity was measured in AM and PM. The level of membrane 5'-nucleotidase activity is inversely proportional to the degree of activation of macrophages (13). That resident AM were more activated than resident PM was also supported by the significantly lower levels of 5'-nucleotidase activity found in resident AM than in resident PM in our studies.

Functionally, AM induced by BCG and recovered by lavage with fluid containing lidocaine were phagocytic and suppressed the in vitro plaque-forming response to the same extent as did unstimulated, resident AM. Although stimulation with BCG resulted in an increase in the absolute number of AM as well as an increase in the proportion of large granular cells obtained by lung lavage, it did not change the relative distribution of cells which stained positively for nonspecific esterase, a marker associated with mature macrophages (8), as compared with controls. Unfortunately, the data presented here cannot differentiate between recruitment followed by maturation or in situ proliferation to account for the increased number of cells recovered in response to BCG.

In conclusion, we showed that the infusion of sonified heat-killed BCG into the tail vein of mice induced large numbers of AM. Further, the addition of lidocaine to the lavage fluid increased the number of AM recovered without affecting the functional and cytochemical characteristics of the AM, thus permitting definitive studies to be carried out on the important immunoregulatory functions of these cells.

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