Role of Activation in Alveolar Macrophage-Mediated Suppression of the Plaque-Forming Cell Response

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Alveolar macrophages (AM) are highly suppressive of the in vitro plaque-forming cell (PFC) response of spleen cells obtained from mice primed with sheep erythrocytes. Comparison of macrophage populations obtained from disparate anatomical sites revealed that although in both cases there was a cell-concentration-dependent suppression of the PFC response, resident AM or AM activated as a result of intravenous injection of Mycobacterium bovis BCG were equally suppressive of the PFC response than were resident macrophages. In contrast, splenic macrophages at comparable concentrations were not at all suppressive. Resident AM exhibited significantly lower levels of S'-nucleotidase activity than did resident peritoneal macrophages. Macrophage-mediated suppression of the in vitro PFC response could not be attributed to the release of toxic oxygen metabolites (H2O2, O2−, and ·OH) or prostaglandins, since the addition of catalase, superoxide dismutase, 2-mercaptoethanol, or indomethacin did not completely reverse suppression. These results suggest that the lung microenvironment may maintain AM in an activated state which contributes to their potential immunoregulatory functions.

By virtue of their nonspecific phagocytic and microbicidal activities, alveolar macrophages (AM) protect the lung against microbial pathogens and environmental pollutants (7, 13). When challenged with a variety of stimuli, AM exhibit immunoregulatory functions (12, 16) which result either in stimulation (11, 18, 19, 26, 33) or suppression (1, 10, 15, 26) of cell-mediated and humoral immune responses. In addition, AM are relatively inefficient in providing accessory function for the initiation of immune responses. This defect is associated with their inability to take up particulate antigens (33), to degrade antigens to an immunogenic form (30), and to effectively interact with T lymphocytes (21, 28) when compared with peritoneal macrophages (PM).

Immunosuppression mediated by macrophages has been attributed to several mechanisms, including the production of nonspecific inhibitors such as arachidonic acid metabolites (4, 8, 24), oxygen metabolites (29, 31), and soluble immune response suppressor-induced factors (2). The hyperoxic condition of the lung microenvironment particularly favors the production of these toxic metabolic end products. AM derive their energy from oxidative metabolism in contrast to PM, which derive their energy from glycolysis; AM also have a respiratory rate three times greater than that of PM (25). As a consequence of constant exposure to airborne and blood-borne substances, resident AM exist in a naturally activated state as indicated by elevated levels of acid phosphatase activity (22) and by their spontaneous production of interleukin-1 (17).

We have previously shown that both resident and Mycobacterium bovis BCG-activated AM suppress the plaque-forming cell (PFC) response (22). In the present study, the roles of activation and possibly of the lung microenvironment in AM-mediated suppression were evaluated by comparing AM with macrophages obtained from other anatomical sites after in vivo activation with BCG. Oxygen metabolites and prostaglandins were also evaluated for their potential role as mediators of AM-induced suppression of the humoral immune response.

MATERIALS AND METHODS

Mice. Certified virus-free BALB/c and C3H/HeJ mice were purchased from Harlan Sprague-Dawley, Inc. (Walkersville, Md.) and the Jackson Laboratory (Bar Harbor, Maine), respectively. The animals were maintained at the Georgetown University Research Resources Facility in accordance with National Institutes of Health guidelines. They were provided with food and water ad libitum.

Collection of macrophages. Murine AM were induced by intravenous injection of heat-killed BCG (22) and collected by bronchoalveolar lavage (23). Briefly, lipopolysaccharide-heat-killed BCG (obtained from Quentin Myrvik, Bowman-Gray School of Medicine, Winston-Salem, N.C.), was homogenized in phosphate-buffered saline (PBS) at 1 mg/ml by ultrasonic probe sonication (Heat Systems Ultrasonics, Inc., Plainview, N.Y.) in an ice bath, and 0.5 ml of the homogenate was injected in the tail veins of 7- to 10-week-old BALB/c mice. Bronchoalveolar lavage was performed 6 to 8 days later with warm (37°C) Ca2+-Mg2+-free PBS containing 17 mM lidocaine hydrochloride (Xylocaine; Astra Pharmaceutical Products Inc., Framingham, Mass.). Bronchoalveolar cells were immediately placed on ice, washed three times, and counted in a hemacytometer. The bronchoalveolar cells consisted of 90 to 93% macrophages, 5 to 10% lymphocytes, and 1 to 2% polymorphonuclear leukocytes as determined by Wright staining (Diff-Quik; American Scientific Products, Inc., McAlpin & Park, Ill.) cytocentrifuge preparations (22).

Peritoneal exudate macrophages (i.e., PM) were induced by intraperitoneal injection of 0.5 mg of BCG cells in 0.5 ml of PBS. Peritoneal exudate cells (75 to 85% macrophages) were recovered 6 to 8 days later by washing the peritoneal cavity with warm lidocaine-containing PBS.

Further enrichment of AM or PM, when necessary, was
achieved by one-step adherence on plastic dishes for 2 to 24 h at 37°C followed by removal of nonadherent cells. This procedure usually resulted in recovery of 95 to 98% viable macrophages, as indicated by their nonspecific esterase activity (22). The macrophages induced with BCG are considered to be activated compared with resident macrophages recovered from untreated normal animals.

Spleenic macrophages were obtained as follows. Tissue culture dishes (no. 3100; Costar, Cambridge, Mass.) were coated with fetal bovine serum at 37°C for 2 h and the serum was decanted. Spleen cells, suspended in complete medium at 107 cells per ml, were added to the dishes and incubated at 37°C in humidified air containing 5% CO2 for 2 h. Nonadherent cells were removed by washing with warm PBS. Adherent macrophages were recovered after an additional 15 min of incubation with 17 mM lidocaine in PBS followed by gentle scraping.

**Macrophage-mediated suppression of the in vitro PFC response.** Spleen cells (107), obtained from BALB/c mice immunized 4 days earlier with sheep erythrocytes (SRBC; Waltz Farm, Smithsburg, Md.) by intraperitoneal injection of 0.2 ml of 10% (vol/vol) SRBC in PBS, were incubated with 2 x 106 SRBC with or without 106 additional macrophages (AM or PM) in a total volume of 1 ml RPMI-fetal calf serum, which consisted of RPMI 1640 (Hazleton Research Products Inc., Denver, Pa.) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, Utah), 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 2 mM L-glutamine (Hazleton Research Products). The cultures were incubated in 24-well tissue culture plates (no. 3424; Costar) at 37°C in 5% CO2 and humidified air. The cultures were harvested after 3 days, and the number of cells secreting anti-SRBC antibodies was determined in a hemolytic plaque assay as previously described (26). The degree of suppression of the PFC response in cultures containing macrophages was calculated in relation to the antigen-stimulated control cultures.

**Treatment of cell cultures.** Thymol-free catalase from bovine liver, superoxide dismutase from bovine erythrocytes, 2-mercaptopethanol, and indomethacin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Prostaglandin E2 (PGE2) and PGE1 were generously provided by Peter W. Ramwell (Georgetown University Medical School, Washington, D.C.). Catalase and superoxide dismutase were dissolved in complete medium at 2 x 10-4 U/ml and 200 µg/ml, respectively. 2-Mercaptopethanol was diluted in distilled water to give a 5 x 10-2 M solution. Indomethacin, PGE1, and PGE2 were solubilized in absolute ethanol and diluted in RPMI 1640. The filter-sterilized reagents were added to the PFC culture at the concentrations indicated.

**Determination of 5'-nucleotidase activity.** 5'-Nucleotidase, a plasma membrane enzyme, was measured by using [3H]AMP (Amersham Corp., Arlington Heights, Ill.) in 54 mM Tris buffer (pH 9.0) containing 12 mM MgCl2 (3). γ-Nitrophenyl phosphate (5.5 mM) was added as a competitive inhibitor of endogenous phosphatase activity. The assay mixture, consisting of 50 µl of macrophage lysate and 0.25 ml of substrate, was incubated at 37°C in a shaking water bath for 30 min. The reaction was stopped by the addition of 0.1 ml of 0.25 M ZnSO4 followed by 0.25 M Ba(OH)2 to precipitate the unreacted substrate. After centrifugation at 600 x g for 30 min at 0 to 4°C, 0.25 ml of the supernatant was added to 5 ml of Aquasol (New England Nuclear Corp., Boston, Mass.), and the level of radioactivity was determined by scintillation counting. Enzyme blanks (no substrate) and standards (100% hydrolysis) were included in each assay. Activity was expressed as units per minute per milligram of protein.

Protein was determined by the procedure of Lowry et al. (20).

**Data analysis.** Paired t tests of differences between means of various groups were performed by using the Tukey studentized range test as described in the Statistical Analysis System computer software (SAS Institute, Cary, N.C.).

**RESULTS**

Effect of resident and activated macrophages on the PFC response. Figure 1 shows the results of experiments in which the effects of adding either resident AM (SRAMN) and PM (SRPMMN) or AM and PM activated by in vivo administration of BCG (SRAMACT and SRPMACT, respectively) on the PFC response of spleen cells obtained from mice previously primed with SRBC were compared. Resident and activated AM were equally effective in suppressing the PFC responses of SRBC-stimulated spleen cell cultures (SR) by greater than 90% when AM were added at a 1:10 ratio with respect to spleen cells. On the other hand, BCG-activated PM were significantly more suppressive than were resident PM (90% versus 51%, P < 0.0001) when added at the same ratio. It should be noted that, under similar conditions, resident splenic macrophages (SRSMN) were not suppressive. Cell-concentration-dependent suppression of the PFC response was observed with both AM and PM. Addition of resident AM (SRAMN) at final concentrations of 5, 1, or 0.1% with respect to spleen cells resulted in the production of 450, 700, and 2,400 PFC/106 cells, respectively. Antigen-stimulated spleen cell cultures to which BCG-activated AM were added (SRAMACT) at final concentrations of 5, 2, or 0.1% produced 300, 550, and 1,900 PFC/106 cells, respectively (data not shown). The differences between suppression induced by BCG-activated and resident AM at the various cell ratios was not statistically significant as determined by the Student t test. A similar cell-concentration-dependent suppression on the PFC response was observed with resident and BCG-activated PM (data not shown).

To determine whether previous exposure of AM to SRBC in vivo was a requirement for suppression, the abilities of

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**FIG. 1.** Immunoregulatory activities of macrophages obtained from different anatomical locations. Immunized spleen cells (107) were cultured alone (S), or with 2 x 106 SRBC (SR), or with SRBC plus 106 normal AM (SRAMN), BCG-activated AM (SRAMACT), normal PM (SRPMN), BCG-activated PM (SRPMACT), or normal SM (SRSMN), and incubated for 3 days. PFC were assayed 3 days after culture initiation and are expressed as PFC/106 cells ± standard error of the mean (SEM) for 6 to 10 determinations.
AM obtained from mice immunized parenterally with SRBC and of resident AM obtained from normal, unimmunized animals to suppress the in vitro PFC response were compared. AM obtained from immunized mice (normal resident AM) were as suppressive as those obtained from immunized mice (Table 1).

**Role of oxygen metabolites.** Since resident AM appear to exist in a more-activated metabolic state than PM (25) as indicated by higher acid phosphatase (22) and lower 5'-nucleotidase (Fig. 2) activities, we were interested in determining whether oxygen metabolites such as H₂O₂, O₂⁻, or ·OH, which are produced by activated macrophages, could account for AM-mediated suppression of the PFC response. As shown in Table 2, the addition of catalase (10³ U/ml) or superoxide dismutase (5 µg/ml) stimulated the PFC response in control (SR) cultures by 8 and 25%, respectively, but had no effect on the suppression of the PFC response in cultures containing AM (SRAM). When superoxide dismutase was added at 10 µg/ml, there was a partial reversal of the suppression mediated by AM (65% in treated versus 92% in control cultures, \( P < 0.005 \)). Addition of higher concentrations of superoxide dismutase (up to 30 µg/ml) did not result in further reversal of AM-induced suppression (data not shown). On the other hand, whereas 2-mercaptoethanol, which is purported to prevent the generation of hydroxyl radicals (·OH) from H₂O₂ and O₂ by increasing the availability of reduced glutathione (14), stimulated the PFC response of control (SR) cultures by almost twofold, it also did not abrogate AM-mediated suppression of the PFC response.

**Comparison of 5'-nucleotidase activity in resident and activated macrophages.** The level of 5'-nucleotidase activity in macrophages has been reported to vary inversely with their state of activation (9). To compare the state of activation in resident and BCG-induced macrophages, cell-free lysates of the various macrophage populations were prepared and assayed for enzyme activity. Several observations can be made from the results presented in Fig. 2. First, resident PM demonstrate a significantly higher level of 5'-nucleotidase activity than do their activated PM counterparts. Second, resident AM demonstrate a significantly lower level of activity of this enzyme compared with resident PM. Third, there is no significant difference in the activity of 5'-nucleotidase in resident AM and BCG-induced AM populations. These results suggest that AM exist in a naturally activated state.

**TABLE 1.** Comparison of suppressive activity of AM from SRBC-immunized and normal mice

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>AM type</th>
<th>PFC/10⁶ cells ± SEM</th>
<th>% Suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>AM</td>
<td>133 ± 8</td>
<td></td>
</tr>
<tr>
<td>SR</td>
<td>SRBC immunized</td>
<td>3,349 ± 286</td>
<td></td>
</tr>
<tr>
<td>SRAM</td>
<td>Normal resident</td>
<td>149 ± 28</td>
<td>95.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56 ± 8</td>
<td>98.3</td>
</tr>
</tbody>
</table>

* Spleen cells (10⁶) from immunized mice were incubated alone (S), with 2 × 10⁶ SRBC (SR), or with SRBC and 10⁶ AM (SRAM) obtained from immunized or nonimmune animals for 3 days, after which the cultures were assayed for PFC.

**TABLE 2.** Effect of addition of catalase, superoxide dismutase, and 2-mercaptoethanol on AM-mediated suppression of PFC response

<table>
<thead>
<tr>
<th>Treatment and culture conditions</th>
<th>PFC/10⁶ cells ± SEM</th>
<th>% Suppression of enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>94 ± 13</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>3,019 ± 131</td>
<td></td>
</tr>
<tr>
<td>SRAM</td>
<td>244 ± 34</td>
<td>91.9e</td>
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<tr>
<td>Catalase (10² U/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>109 ± 16</td>
<td>8d</td>
</tr>
<tr>
<td>SR</td>
<td>3,279 ± 152</td>
<td></td>
</tr>
<tr>
<td>SRAM</td>
<td>190 ± 47</td>
<td>94.2e</td>
</tr>
<tr>
<td>Superoxide dismutase (5 µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>95 ± 19</td>
<td></td>
</tr>
<tr>
<td>SR</td>
<td>3,747 ± 146</td>
<td>25.0e</td>
</tr>
<tr>
<td>SRAM</td>
<td>35 ± 1.0</td>
<td>99.1e</td>
</tr>
<tr>
<td>Superoxide dismutase (10 µg/ml)</td>
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</tr>
<tr>
<td>S</td>
<td>84 ± 11</td>
<td></td>
</tr>
<tr>
<td>SR</td>
<td>3,449 ± 348</td>
<td>14.2e</td>
</tr>
<tr>
<td>SRAM</td>
<td>1,138 ± 131</td>
<td>64.6e</td>
</tr>
<tr>
<td>2-Mercaptoethanol (2.5 × 10⁻³ M)</td>
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<td></td>
</tr>
<tr>
<td>S</td>
<td>25 ± 5</td>
<td></td>
</tr>
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<td>SR</td>
<td>5,205 ± 28</td>
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<td>SRAM</td>
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<td>2-Mercaptoethanol (5 × 10⁻³ M)</td>
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</tr>
<tr>
<td>S</td>
<td>54 ± 5</td>
<td></td>
</tr>
<tr>
<td>SR</td>
<td>5,884 ± 253</td>
<td>94.9e</td>
</tr>
<tr>
<td>SRAM</td>
<td>351 ± 18</td>
<td>94.0e</td>
</tr>
</tbody>
</table>

* Spleen cells (10⁶) from immunized mice were incubated alone (S), with 2 × 10⁶ SRBC (SR), or with SRBC and 10⁶ AM (SRAM) in the presence or absence of catalase (10³ U/ml), superoxide dismutase (5 or 10 µg/ml), or 2-mercaptoethanol (2.5 × 10⁻³ or 5 × 10⁻³ M) for 3 days.

* SEM of 3 to 12 determinations.

* % Suppression due to AM was calculated as [(SR - S) - (SRAM - S)]/(SR - S) × 100.

* % Enhancement was calculated as [(treated SR - untreated SR)/untreated SR] × 100.
FIG. 3. Effect of indomethacin on AM-mediated suppression of the PFC response. Indomethacin (2.8 × 10^{-8} and 4.2 × 10^{-8} M) was added to cultures containing 10^7 immunized spleen cells alone (S), plus 2 × 10^5 SRBC (SR), or plus SRBC in the presence of 10^6 AM (SRAM), and the cultures were incubated for 3 days. The number of PFC was determined and is expressed as PFC/10^6 cells ± SEM for three to six determinations.

**Role of arachidonic acid metabolites.** A series of experiments was carried out to determine whether the production of arachidonic acid metabolites by AM could account for their suppressive effect on the PFC response. In preliminary experiments, the direct addition of PGF_2α or PGF_2β at concentrations from 10^{-4} to 10^{-6} M resulted in suppression of the PFC response of control (SR) cultures by greater than 90%, demonstrating the immunosuppressive activity of these metabolites in our immunological assay system (data not shown). That PGF_2α or PGF_2β was not responsible for the AM-mediated suppression of the PFC response can be seen in the results of experiments presented in Fig. 3. Addition of indomethacin to SRAM cultures at concentrations of 2.8 and 4.2 × 10^{-8} M, which have been reported to inhibit prostaglandin synthetase activity (32), resulted in a slight increase in the absolute number of PFC in all SRAM cultures, but only marginally reduced AM-mediated suppression of the PFC response when compared with untreated SRAM cultures (64 and 80% suppression with indomethacin-treated cultures versus >90% suppression in untreated SRAM cultures).

**DISCUSSION**

Several studies have demonstrated the immunosuppressive activities of alveolar macrophages (1, 10, 15, 26); however, the possible mechanisms involved in this important immunoregulatory function have not been vigorously evaluated. In contrast, immunosuppression associated with PM has been attributed to the production of toxic oxygen metabolites such as hydrogen peroxide (5, 24), superoxide anion (6), and prostaglandins (4, 8, 24), as well as to a combination of the two substances produced by these cells (18). It would not be unreasonable to assume that AM can produce large quantities of toxic oxygen metabolic end products since, in the hyperoxic lung microenvironment, these cells depend to a large degree on oxidative metabolism to derive energy, and as a consequence they exhibit a respiratory rate three times greater than that of peritoneal macrophages (25). In addition, because of their unique location, AM are constantly exposed to infectious agents, allergens, and environmental pollutants; therefore, their scavenger functions are likely to keep them in a highly activated state (12).

Recently, we have presented evidence which suggests that resident AM exist in a more activated state relative to resident PM as indicated by the detection of higher levels of cytoplasmic acid phosphatase activity in resident AM (22). In the present study, we have confirmed and extended our previous observations by demonstrating that in resident AM there is a lower level of 5'-nucleotidase activity, a plasma membrane enzyme whose level is inversely related to the level of macrophage activation (9). Since the peritoneal macrophages used in studies which demonstrate their immunoregulatory capabilities were elicited with thioglycolate, peptone, or other agents, it is possible that they were immunosuppressive by virtue of their activated state upon collection. We have shown herein (Fig. 1) a correlation between activation and suppression by AM and PM. Since resident AM (Fig. 2) are relatively activated, one can assume that the lung microenvironment maintains these cells in an activated state which contributes to their immunosuppressive capabilities.

Oxygen metabolites (H_2O_2, O_2^-, and ·OH) elaborated by AM could not account entirely for AM-mediated suppression, since addition of either catalase or superoxide dismutase could not abrogate suppression. The slight decrease in AM-mediated suppression resulting from treatment with a 10-µg/ml concentration of superoxide dismutase indicates that superoxide anion may play a minor contribution to suppression. These results were supported by the observation of an overall stimulation of control PFC responses after the addition of 2-mercaptoethanol, with no accompanying effect on AM-mediated suppression. 2-Mercaptoethanol prevents the generation of toxic hydroxyl radicals from hydrogen peroxide reacting with oxygen by increasing the availability of reduced glutathione, which reduces H_2O_2 to water (14).

Our data also suggest that prostaglandins do not appear to play a significant role in AM-mediated suppression, since treatment of cultures with indomethacin reduced AM-mediated suppression by only 30%, even though the addition of PGF_2α and PGF_2β caused significant suppression of the PFC response in control cultures. These results are not entirely consistent with the observations of Demenkov et al. (8), who reported that canine AM suppressed mitogen-stimulated lymphoproliferation through the elaboration and release of high levels of indomethacin-inhibitable factors, including prostaglandin. This discrepancy may be attributed to differences in the animal species studied or in the immunological assay employed (12).

In other studies, we have presented evidence which suggests that AM membrane sialoglycoconjugates play an important role in AM-mediated suppression. In that study (21), generation of aldehydes on AM membrane sialoglycoconjugates as a result of treatment with sodium periodate resulted in total reversal of suppression of the PFC response. More recently, we observed that membrane gangliosides suppressed the PFC response of SRBC-primed spleen cells in a dose-dependent manner. Addition of rabbit anti-mouse brain antiserum, which reacts with the gangliosides, inhibits both ganglioside- and AM-mediated suppression of the PFC response. Pretreatment of AM, but not spleen cells, with the antganglioside antibody inhibits AM-mediated suppression, which suggests that secreted membrane gangliosides play an important role in AM-mediated suppression of the PFC response (I. N. Mbawuike and H. B. Herscowitz, Cell. Immunol., in press). These results are supported by similar studies which showed that membrane extracts from activated peritoneal exudate macrophages were cytostatic for...
tumor target cells (27). The active components were shown to be N-acetylneuraminic acid-containing glycosphingolipids (gangliosides). One can, therefore, speculate that the lung microenvironment maintains AM in a relative activated state, resulting in physiological changes, such as increased expression of membrane gangliosides, which may contribute to an alteration of their potential immunoregulatory activities.

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LITERATURE CITED


