Induction of in Vitro Tumoricidal Activity in Alveolar Macrophages and Monocytes from Patients with Lung Cancer

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ABSTRACT

Human alveolar macrophages (AMs) and blood monocytes were obtained from 65 smoking and nonsmoking normal volunteers and 29 patients with lung cancer. The oxidative metabolic response of these cells was measured by superoxide anion production after incubation with lipopolysaccharide. In addition, tumoricidal activity of AMs and monocytes was assessed against [3H]thymidine-labeled tumor target cells. Smoking was associated with depressed AM superoxide anion responses in normals but not in patients. In contrast, smoking appeared to slightly elevate monocyte superoxide anion activity. AMs and monocytes exposed to lipopolysaccharide or recombinant γ-interferon showed tumoricidal activity in all groups. Mean cytotoxicity values of smoking patients versus smoking normals and exsmoking patients versus nonsmoking normals were not significantly different. Smoking, however, in both patients and normals was associated with significantly (P < 0.005) depressed AM cytotoxicity levels (<40%) compared to nonsmoking volunteers and exsmoking patients. Activated AMs from cancer patients and normals were cytotoxic against three different tumorigenic cell lines but not against a nontumorigenic line. No correlation between monocyte and AM cytotoxic activity within single individuals was found. We conclude that AM and monocytes from smoking and exsmoking patients can be activated after exposure to immunomodulators; however, smoking may be slightly suppressive to cytotoxic responses. These studies provide a rationale for clinical trials of immunomodulators in patients with lung cancer.

INTRODUCTION

Bronchogenic carcinoma is the most common fatal cancer in this country (1). Currently available therapy is inadequate and the 5-year survival rate in lung cancer is approximately 10% (1). In vivo and in vitro studies have suggested an anticancer role for macrophages. Rodent and human macrophages activated in vitro by immunomodulators (LPS, γ-interferon, C-reactive protein, lymphokines, colony-stimulating factors) acquire the ability to kill neoplastic cells (2–6). In several animal tumor model systems, immunomodulators have been shown to reduce metastases and increase survival (7–9). Information obtained from these animal studies suggests that alveolar macrophages are important in immune surveillance and can be stimulated to be cytotoxic for tumors metastatic to the lung. Such animal studies raise the possibility that monocytes/macrophages of patients with lung cancer could be activated in situ to the tumoricidal state by systemically administered immunomodulators and thus enhance host-mediated destruction of tumor cells. Justification of clinical trials of macrophage-activating agents requires assurance that alveolar macrophages and blood monocytes of patients with lung cancer can respond to activating agents in vitro. Furthermore, in vitro studies may aid in the selection of patients for clinical trials and in the evaluation of therapy.

The purpose of the present study was to examine: (a) the response to activating agents of alveolar macrophages and blood monocytes from patients with lung cancer with regard to the oxidative metabolism and tumoricidal activity; and (b) the effect of smoking status and lung cancer on the response to activating agents.

MATERIALS AND METHODS

Normal Volunteers. Alveolar macrophages and peripheral blood monocytes were obtained from 65 normal healthy volunteers; 31 were nonsmokers (mean age, 27 ± 8 [SD] years) and 34 were cigarette smokers (mean age, 35 ± 10 years). The mean cigarette consumption by smokers was 20 ± 20 pack-years (median, 14 pack-years) with a range of 2–113 pack-years. Due to limitations in cell yields, not all assays were performed with each individual's cells.

Patient Population. Alveolar macrophages and monocytes were obtained from 29 patients with primary lung cancer (mean age, 62 ± 9 years). None of the patients had received prior chemotherapy or radiation for his or her disease. All of the patients had negative bacterial cultures from their bronchial washings. The mean cigarette consumption was 45 ± 24 pack-years (median, 44 pack-years) with a range of 2–111 pack-years. Fourteen patients were active smokers, 13 had quit 1 year or more prior to diagnosis, and 2 had never smoked. Of the 4 patients with small cell carcinoma, 2 had extensive disease and 2 had limited disease. The 25 patients with non-small cell carcinoma were staged as follows: Stage I, 10; Stage II, 3; Stage IIIa, 8; and Stage IV, 4 (10). Only 3 had known metastatic disease at the time of bronchoscopy. The cell differentials from the bronchial lavages of patients (92 ± 8% alveolar macrophages) were not different from those of the normal volunteers (95 ± 4% macrophages for smokers; 93 ± 5% for nonsmokers).

Preparation of Alveolar Macrophages. Alveolar macrophages were obtained by fiberoptic bronchoscopy as previously described (11). Patients were lavaged on the contralateral side to their tumor. All lavage fluid was passed through a blood filter (McCaw, Sabana Grande, PR) and then centrifuged at 400 × g for 10 min. The cell pellet was then washed three times with HBSS. Cell number was determined on a hemocytometer and differential cell counts were performed with either a modified Wright's stain (Diff-Quik; American Scientific, McGaw Park, IL) or a nonspecific esterase stain (12). The cells were resuspended in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 5% human AB serum (Hazelton, Denver, PA) and L-glutamine. The cell concentration was adjusted to 5 × 10⁶/ml and 0.1 ml was added to 96-well microtiter plates (Falcon Laboratories, McLean, VA). Cells were allowed to adhere for 60 min at 37°C in a moist 5% CO₂ incubator, and nonadherent cells were then removed by washing the wells with warmed RPMI. By nonspecific esterase (Sigma, St. Louis, MO) cytochemistry, over 99% of adherent cells were characterized as macrophages. Endotoxin contents of culture reagents did not exceed 0.3 ng/ml as determined by the Limulus amebocyte assay (Associates of Cape Cod, Inc., Woods Hole, MA).

Preparation of Peripheral Blood Monocytes. Mononuclear leukocytes were isolated from heparinized whole blood by centrifugation over LeucoPREP (Becton Dickinson, Lincoln Park, NJ) and were resuspended in complete RPMI 1640. Cell number and differential cell counts were determined as described above for macrophages. The
monocyte concentration was adjusted to $1 \times 10^7$/ml and 0.1 ml was added to 96-well microtiter plates. Cells were adhered as described above for macrophages. By nonspecific esterase cytochemistry, over 95% of adherent cells were characterized as monocytes.

**Superoxide Anion Assay.** Superoxide anion production was measured in an enzyme immunoassay microplate reader as previously described (13, 14). Microtiter plates with adherent macrophages or monocytes were incubated with or without *Salmonella typhimurium* lipopolysaccharide (Difco, Detroit, MI). In initial experiments, macrophages were incubated with LPS for 1–4 days. The peak superoxide anion response occurred on the fourth day. Subsequently, all plates were incubated 4 days. After incubation, the plates were gently washed with phenol red-free HBSS and each well received 100 µl HBSS containing 16 nmol ferricytochrome $c$, with 1 nmol phorbol myristate acetate to trigger the release of reactive oxygen species. Superoxide anion production was inhibited totally by 600 units superoxide dismutase/well. Production of superoxide anion was detected by measuring absorbance of reduced ferricytochrome $c$ at 550 nm.

A qualitative grading system was used to ensure that adherent cell numbers present in monolayers after culture for 4 days were not different from each other. This system was established by comparing estimated cell numbers to actual cell number determinations. Monolayers were stained with crystal violet and then cell number was determined in four replicates by counting cells with an inverted microscope at $\times 400$. Grades were as follows: good, >100 cells/field; fair, 75–100 cells/field; and poor, <75 cells/field. Only results from monolayers classified as good were included. Cell numbers did not differ between cultures from smokers and nonsmokers.

**Tumoricidal Assay.** The tumoricidal assay is based on that described by Kleinerman et al. (5). Adherent monocytes or macrophages were cultured in microtiter plates as described above. The cells were incubated in the presence or absence of LPS or recombinant γ-interferon (specific activity, 2.2 × 107 units/mg; Biogen, Cambridge, MA) and, after 20 h, were washed with warm medium. Target cells previously labeled for 24 h with 5 µCi/ml tritiated thymidine (specific activity, 5 Ci/mmol); New England Nuclear, Boston, MA) were added at a target to effector ratio of 1:10. In preliminary experiments, ratios were varied from 1:5 to 1:50 and maximal tumoricidal activity was demonstrated at a 1:10 ratio. Target cells were cocultured with macrophages or monocytes for 24–96 h, after which time the trays were gently washed with normal saline to remove nonadherent cells. The nonadherent cells found in the supernatant after coculture were less than 5% viable as determined by trypan blue exclusion. Residual adherent cells were lysed with 0.1 N NaOH and aliquots of lysate were added to scintillation fluid for determination of radioactivity. The cytotoxic activity of the macrophages/monocytes was calculated as follows:

\[
\text{% of cytotoxicity} = \frac{\text{dpm in target cells cultured with } \text{LPS-treated macrophages/monocytes} - \text{dpm in target cells cultured with control macrophages/monocytes}}{\text{dpm in target cells cultured with control macrophages/monocytes}} \times 100
\]

Endogenous (spontaneous) % of cytotoxicity
\[
\text{dpm in target cells in medium alone} - \text{dpm in target cells cultured with untreated macrophages/monocytes} \times 100
\]

**Target Cells.** Target cells included: SK-MEL-28 (melanoma) and CDD-11 Lu (nonneoplastic lung fibroblasts), obtained from the American Type Culture Collection (Rockville, MD); and CRL 1718 (astrocytoma), as previously described (5). CCF-RC-1, a renal carcinoma cell line, was obtained from Dr. J. Edson Pontes of our institution. Target cells were cultivated in RPMI 1640 supplemented with 10% fetal calf serum and were free of *Mycoplasma* and other microbial contaminants.

**Statistical Analysis.** Statistical significance of data was determined by comparing results of smoking normal volunteers to smoking patients and results of nonsmoking normal volunteers to exsmoking patients in various assays with 2-tailed Student's $t$ test (unless indicated otherwise in "Results"). Correlation of monocyte with macrophage cytotoxicity was analyzed with Pearson product-moment method (15, 16), and comparison of the effects of smoking versus the distribution of cytotoxicity values was analyzed by $\chi^2$.

**RESULTS**

**Superoxide Anion Production by Alveolar Macrophages and Blood Monocytes from Patients and Normal Volunteers.** Accumulated superoxide anion production over 180 min by monocytes and alveolar macrophages from smoking and nonsmoking normal volunteers and patients is shown in Fig. 1. After culture for 4 days with LPS, PMA-treated nonsmoker macrophages had significantly greater superoxide anion production than macrophages from normal volunteer smokers ($P = 0.001$), but were not different from nonsmokers. No correlation between number of pack-years and superoxide anion response could be demonstrated. Both smoker and nonsmoker macrophages had greater superoxide anion production than monocytes in response to LPS activation. Monocytes from normal volunteer smokers and nonsmokers were not significantly different in superoxide anion production. However, monocytes from patients (3 smokers, 2 exsmokers) produced more superoxide anion than those from nonsmokers ($P = 0.01$).

**Tumoricidal Activity of Alveolar Macrophages and Blood Monocytes from Patients and Normal Volunteers.** Tumoricidal activities against SK-MEL-28 of monocytes and macrophages from smokers and nonsmoker normal volunteers and patients were compared (Figs. 2 and 3). Monocytes demonstrated slightly more spontaneous tumoricidal activity than macrophages. Activation of monocyte tumoricidal activity by either LPS or interferon was not affected by smoking history or cancer status. However, LPS-activated macrophages from smoking normals demonstrated slightly less tumoricidal activity than those from nonsmoking normals ($P = 0.04$) (see Fig. 3). No significant differences were noted in any other comparisons by smoking or disease status.

Although mean cytotoxicity values showed insignificant differences, more smoking normals and patients were noted empirically to have macrophage cytotoxicity levels below 40% than nonsmoking normals and patients for both interferon (0 of 17 nonsmoking normals were below 40%; 2 of 8 exsmoking patients; 6 of 18 smoking normals; 6 of 10 smoking patients; $\chi^2 = 10.15, P < 0.005$), and LPS (2 of 21 nonsmoking normals; 2 of 8 exsmoking patients; 12 of 29 smoking normals; 5 of 11 smoking patients; $\chi^2 = 7.97, P < 0.005$).

No correlation between monocyte and macrophage cytotoxic activity within single individuals was found. In addition, no apparent correlation between number of pack-years and cytotoxicity could be demonstrated. The effect of donor age on macrophage cytotoxicity was also evaluated. The cytotoxicity results of macrophages from 9 older smoking normal volunteers (mean age, 49 ± 8 years; range, 40–64 years) were compared to those of 13 younger smoking volunteers (mean age, 27 ± 1 years; range, 25–29 years). No difference for LPS-stimulated cytotoxicity [40.2 ± 4.0 (SE) versus 46.3 ± 6.0] or for interferon [58.3 ± 3.6 versus 48.3 ± 6.6] was found between the two age groups.

Cytotoxicity responses of alveolar macrophages and monocytes from patients were not dependent on stage of disease. Macrophages from all 20 patients tested with LPS had re-
Fig. 1. Superoxide anion production by monocytes (left) and alveolar macrophages (right) from normal volunteers and patients with lung cancer. Phorbol myristate acetate stimulation and superoxide anion measurements were carried out on day 4 after cell culture in medium with or without 5 μg/ml S. typhimurium lipopolysaccharide. Columns, mean nmol superoxide anion accumulated/10⁶ cells at 180 min. Bars, SEM.

Fig. 2. Cytotoxicity of LPS- or IFN-activated monocytes from normal volunteers and patients. Adherent monolayers of monocytes were incubated for 20 h in control medium or with either 5 μg/ml S. typhimurium lipopolysaccharide or 1000 units/ml of IFN; they were then washed and cocultured for 72-96 h with [³H]thymidine-labeled target cells (SK-MEL-28). Points, results from each individual donor for medium control (spontaneous cytotoxicity) and LPS- or IFN-activated cytotoxicity. O, normal volunteer nonsmoker; ●, normal volunteer smoker; ◦, lung cancer patient exsmoker; ●, lung cancer patient smoker; *, lung cancer patient nonsmoker. ---, mean cytotoxicity value for each group.

Fig. 3. Cytotoxicity of LPS- or IFN-activated alveolar macrophages from normal volunteers and patients. Adherent monolayers of macrophages were incubated for 20 h in control medium or with either 5 μg/ml S. typhimurium lipopolysaccharide or 1000 units/ml of IFN; they were then washed and cocultured for 72-96 h with [³H]thymidine-labeled target cells (SK-MEL-28). Points, results from each individual donor for medium control (spontaneous cytotoxicity) and LPS- or IFN-activated cytotoxicity. O, normal volunteer nonsmoker; ●, normal volunteer smoker; ◦, lung cancer patient exsmoker; ●, lung cancer patient smoker; *, lung cancer patient nonsmoker. ---, mean cytotoxicity value for each group.

Responses greater than 20% cytotoxicity and only 1 of 19 did not respond to interferon. Monocytes of 5 of 16 patients tested with LPS had responses of less than 20%. Monocytes from all 15 patients tested with interferon responded (including the 5 who failed to respond to LPS).

Spectrum of Tumoridical Activity of Activated Alveolar Macrophages. Macrophages demonstrated little or no spontaneous tumoridical activity against any of the 3 target cell lines (Fig. 4). However, LPS- or interferon-activated macrophages from all groups demonstrated cytotoxicity against the tumorigenic
Cancer patient exsmoker; lung cancer patient smoker. Lung lavages of these patients tended to be lower. Monocytes or with either $\text{S}_{\text{S}} \text{g/ml} \text{S. typhimurium lipopolysaccharide}$ or $1000 \text{units/ml IFN-\text{c}}$; they were then washed and cocultured for 72-96 h with $\text{H}$thymidine-

DISCUSSION

The present study demonstrates that alveolar macrophages and peripheral blood monocytes from patients with lung cancer respond in vitro to immunomodulators and acquire the ability to selectively kill tumor cells. Furthermore, smoking may have an effect on these abilities.

Alveolar macrophages from nonsmoking normals and smoking patients were significantly more metabolically active in response to LPS exposure than those from smoking normals. Superoxide anion production of alveolar macrophages was not measured in exsmoking patients, because cell yields from bronchial lavages of these patients tended to be lower. Monocytes from patients (although not analyzed according to smoking history) tended to have higher superoxide anion production than monocytes from smoking and nonsmoking volunteers. The mean induced cytotoxic activity of both monocytes and macrophages was not different for smoking patients versus smoking normals and exsmoking patients versus nonsmoking normals. Smoking tended to be associated with lower metabolism.

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Fig. 4. Specificity of alveolar macrophage cytotoxicity. Adherent macrophages from normal volunteers and patients were incubated for 20 h in control medium or with either 5 μg/ml S. typhimurium lipopolysaccharide or 1000 units/ml of IFN-γ; they were then washed and cocultured for 72–96 h with [3H]thymidine-

and it is possible that more than one mode of action is operative depending on intrinsic macrophage/monocyte properties, local environment, and target cell properties. The higher level of superoxide anion release from alveolar macrophages of nonsmoking normals compared to smoking normals would suggest that if tumor cell killing was occurring by this mechanism alone, macrophages from nonsmokers should have much greater cytotoxic activity. Since PMA is necessary to trigger the release of superoxide anion after activation with LPS, and because PMA was not present in the tumoricidal assay system, we may be examining different manifestations of cell activation with these two assays. The higher level of superoxide anion release in macrophages from smoking patients in comparison to those from smoking normals suggests that cells from patients may be partially activated. Activation is believed to be a multistep process with certain functional activities (e.g., tumor cell killing) requiring a higher level of activation (18).

In contrast to our results, Weissler et al. (19), using a $\text{S}^{125}$Cr release system, could not demonstrate cytotoxic activity after LPS stimulation with human alveolar macrophages from normal nonsmoking individuals or patients with lung cancer. Controversy exists regarding various cytotoxicity assays and whether similar mechanisms are being measured in different assays (20). We believe that our assay measures a tumor-specific effect because of the failure to detect killing of proliferating normal cells (CCD-11 Lu). In addition, the failure to detect viable cells in assay supernatants indicates that the measured phenomena are not simply due to the lack of adherence of proliferating cells. Furthermore, Kleinerman et al. (5), in a similar cytotoxic assay using $\text{S}^{125}$I- rather than $\text{S}^{3}$H-thymidine-labeled targets, showed that more than 90% of the $\text{S}^{125}$I present in the supernatants was in soluble form and the rest was associated with dead cells and debris. Several other studies (21–23) have examined only the spontaneous cytotoxic activity of alveolar macrophages from lung cancer patients. We found very little spontaneous activity while all of these studies found activity. These studies differed from ours in preparation of alveolar macrophages, target cells used, and assay method, and controls were patients with other pulmonary diseases.

Superoxide anion production and cytotoxic activity of monocytes and alveolar macrophages taken from the same individual did not correlate. These observations suggest that circulating immune cell function is not indicative of pulmonary immune cell function. Similar observations have been made in sarcoidosis (24).

In summary, we have demonstrated that alveolar macrophages and monocytes from patients with lung cancer can be activated to produce increased tumoricidal activity and oxidative metabolites. These studies provide a rationale for future clinical trials using biological response modifiers in the treatment of bronchogenic carcinoma.
ALVEOLAR MACROPHAGE FUNCTION OF LUNG CANCER PATIENTS


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