Rat Lung Macrophage Tumor Cytotoxin Production: Impairment by Chronic in Vivo Cigarette Smoke Exposure

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ABSTRACT

Macrophages in the presence of bacteria-derived lipopolysaccharide (LPS) stimuli produce a soluble cytotoxin which is toxic to tumor cells. In this study, we examined various parameters of cytotoxin production from pulmonary lavage cells obtained from Fisher 344 cesarean-derived rats. Cultures of macrophages were derived from pulmonary lavage cells and stimulated in vitro with LPS. Cytotoxin production was assayed in vitro using an L-929 cell target assay. Pulmonary lavage preparations contained a relatively pure population of macrophages, and adherence studies revealed that nonadherent lavage cells contributed negligible amounts of cytotoxin, indicating that macrophages were responsible for cytotoxin production. After LPS stimulation, cytotoxin production became maximal within 10 h and thereafter plateaued. Doses of LPS above 0.1 µg/ml were optimal for production, and in the absence of LPS, no cytotoxin was detected. Because cigarette smoke is the major etiological factor in the development of lung cancers and because smoking is known to profoundly alter the function of alveolar macrophages in humans and experimental animals, subsequent experiments examined the role of chronic cigarette smoke exposure on tumoricidal activity of lung macrophages. Rats were exposed in vivo for 8 wk to either cigarette smoke or air (sham-treated controls). When lavage cells were cultured and stimulated with LPS (1 µg/ml), 5- to 10-fold less cytotoxin was produced by lavage cells from rats exposed to cigarette smoke. Similarly, using a direct cytotoxicity assay, lung macrophages of smoke-exposed animals also revealed marked impairment in cytotoxicity against L-929 cell targets, and this was noted over a wide range of macrophage:tumor target cell ratios. Another product of macrophages, interferon, was also decreased in rats exposed in vivo to cigarette smoke when compared to sham-treated controls. These results suggest that cigarette smoke exposure may impair pulmonary macrophage-mediated tumor defense mechanisms.

INTRODUCTION

Various researchers have found that cigarette smoke exposure impairs the immune response. This includes humoral (1–3) and cellular (4, 5) immune responses in cigarette-smoking humans and in animals chronically inhaling cigarette smoke. Alveolar macrophage functions in vitro after cigarette smoke exposure show altered chemotactic responsiveness (6), decreased responsiveness to migration inhibitory factor (7), enhanced spontaneous migration (7), and impaired phagolysosome fusion (8). It has not yet been established whether cigarette smoking results in impaired cell-mediated immunity against human lung cancer.

Macrophages have been shown to be important in the control of tumor cell proliferation as reflected by their ability to be cytoidal and cytostatic to tumor cells while being relatively nontoxic to normal cells (9, 10). Using radiolabelled nucleotide incorporation and release in tumor cell targets, human pulmonary macrophages have been shown to be cytoidal to human tumor (11) and human lung tumor cell lines (11, 12). Moreover, several previous studies have shown that macrophage oncolytic activity can, at least in part, be attributed to soluble cytotoxins. This has been demonstrated in macrophages from mice (13, 14), rabbits (15), and humans (16–18). Rabbit pulmonary macrophages have been shown to be good producers of soluble cytotoxin (15). In the mouse (19) and rabbit (15) systems, this cytotoxin has been shown to be similar to tumor necrosis factor.

Recently we have compared several in vitro assays for tumor cytotoxicity measurement (20). Actinomycin D treatment of target cells dramatically increased an assay’s sensitivity. Using an actinomycin D-treated L929 target cell assay, we evaluated the parameters of rat lung macrophage cytotoxin production and investigated whether chronic in vivo cigarette smoke exposure impairs tumoricidal functions of macrophages.

MATERIALS AND METHODS

Animals. Experimental animals used in this study were male Fisher 344 cesarean-derived rats (Charles River Breeding Laboratories, Boston, MA). The rats were free of specific pathogens and were kept isolated from other laboratory animals. They were housed in air-filtered cages and provided with standard laboratory feed and fresh water ad libitum. Histological examination of the lungs when the rats were sacrificed only occasionally revealed minimal degrees of pneumonitis without evidence of infection.

In Vivo Smoke Exposure. Exposure to cigarette smoke was begun when the rats were 6 to 8 wk of age and at least 125 g in weight. Exposure was initiated with one cigarette per day and increased by one-half cigarette per day until three cigarettes per day were reached. This dosage was continued on a Monday through Friday schedule for 8 wk. Cigarettes used in this study were commercial 70-mm nonfiltered cigarettes containing 20.6 mg of tar and 1.42 mg of nicotine per cigarette.

The smoke exposure apparatus was a Maddox-ORNL system, an intermittently puffing machine with puff parameters of 1/min, 2-s duration, and 35-ml volume (21). The puff of smoke was diluted 10:1 with air in the exposure chamber and held in the chamber for 30 s. The chamber was then purged with medical quality air for 28 s before the next puff. During exposure the rats were restrained in conical glass holders that fit into the wall of the exposure chamber, so that only the nose of the animal protruded into the chamber. The sham-exposed animals were handled in the same manner as the experimental animals and went through the same exposure without a cigarette in the cigarette holder. To control for the variability that is inherent in biological systems, we simultaneously paired sham-exposed controls and experimental groups for each individual experiment.
Cigarette-Impaired Macrophage Tumoricidal Activity

Lung Macrophage Harvesting. After 8 wk of exposure, the animals were anesthetized by an i.p. injection of pentobarbital, 50 mg/kg, and killed by exsanguination. Under sterile conditions, the trachea was cannulated with a polyethylene catheter, and lung lavage was performed with sterile isotonic saline.

Cytotoxin and Interferon Production. Lavage cells were centrifuged and suspended in Eagle’s minimal essential medium supplemented with 2× normal concentrations of essential and nonessential amino acids, vitamins, and 4 mM glutamine, 2 mM sodium pyruvate, 250 units of penicillin per ml, 125 μg of streptomycin per ml, 50 μg of gentamycin per ml, and 5% fetal bovine serum. Cells were then enumerated in a hemocytometer and suspended at a density of either 4.0 or 7.5 × 10⁵ alveolar macrophages per ml. Trypan blue exclusion revealed viability to be greater than 95%, and cellular composition was greater than 95% macrophages not varying between treated and sham-treated control animals. One ml of cell suspension was pipetted into wells of 24-well flat-bottomed Linbro tissue culture plates (Flow, Rockville, MD), and Salmonella typhimurium LPS² (Sigma, St. Louis, MO) was added to cultures in the desired concentration. Plates were incubated for 24 h (unless otherwise stated) at 37°C in a 5% CO₂ atmosphere, and culture supernatants were collected, centrifuged free of cells, and either assayed for cytotoxicity or interferon immediately or frozen at −20°C for later assay. Cultures were separately prepared from each rat (cells from multiple rats were never pooled), and for each experiment, control and experimental cultures were run simultaneously (side by side).

Soluble Cytotoxicity Assay. This assay has been described in detail elsewhere (20) but briefly is carried out as follows. Target L929 cells (American Type Culture Collection CCL 1) were seeded into 96-well flat-bottomed Linbro tissue culture plates (Flow, Rockville, MD) and serial 2-fold dilutions were performed with a handheld pipet. Controls contained an infinite dilution of cytotoxin. Actinomycin D with warmed medium. After rinsing, 100 μl of Eagle’s minimal essential medium containing 2× normal concentrations of essential and nonessential amino acids, vitamins, 4 mM glutamine, 5% fetal bovine serum, and antibiotics were added to each well. To wells in Row 1, desired numbers of lavage cells were added in 100 μl of medium, and serial 2-fold dilutions of lavage cells were made with a hand-held pipet. After allowing lavage cells to settle and adhere to target cells for 1 h at room temperature, 100 μl of medium containing 1 μg of LPS (final concentration) per ml were added to each well. Plates were then incubated for 20 h at 37°C in 5% CO₂, removed, stained with crystal violet, rinsed, and dried. Cytotoxicity was enumerated in a similar fashion to that done above in the soluble cytotoxin assays. The percentage of cytotoxicity was calculated for each macrophage:target cell ratio, and lavage cells from each animal were run separately in octuplicate (cells from multiple rats were never pooled). For each experiment, control and experimental lavage cells were run simultaneously (side by side).

Interferon Assay. Rat macrophage interferon was titrated using a plaque reduction assay on Mouse L-929 cells. Although generally considered species specific, cross-species interferon activity has been demonstrated between the rat and mouse (23). L-929 cells, 7.5 × 10⁴, were seeded into 24-well Linbro plates (Flow, Rockville, MD), and serial 2-fold dilutions of macrophage supernatants were added to the wells (final volume, 1 ml). Medium consisted of Eagle’s MEM with 2 mM glutamine, 30 mM HEPES, 10% bovine serum, and antibiotics in the above-mentioned concentrations. Assays done in triplicate were then placed in 37°C for 18 h in a walk-in incubator. Following incubation, supernatants were removed, and cells were infected for 1 h at room temperature with 50 to 100 plaque-forming units of vesicular stomatitis virus in 100 μl of medium. After infection, cultures were overlayed with 1 ml of Eagle’s MEM containing 1% methyl cellulose, 30 mM HEPES, 2 mM glutamine, 5% bovine serum, and antibiotics. After similar incubation for 48 h, plates were stained with crystal violet, rinsed, and dried, and plaques were enumerated. The interferon titer was defined as the reciprocal of the dilution necessary to inhibit plaque formation by 50%. Each animal’s culture supernatants were separately assayed, and control and experimental treatments were run simultaneously (side by side).

Statistical Analysis. As mentioned previously, lavage cells from each individual rat were separately cultured, and resulting supernatants were separately assayed. Cells or supernatants were never pooled. Thus each rat represents one individual event or replicate. Assays for each individual experiment were always run simultaneously with paired controls in order to account for the inherent variability of the bioassay system used. Where shown, titer averages and standard deviations were calculated based on n = number of rats and represent n replicates. Further statistical analysis (P values) were performed by the use of a one-sided Student’s t test for unpaired data.

RESULTS

Parameters of Rat Pulmonary Macrophage Cytotoxin Production

Cytotoxin Production by Adherent Cells. Pulmonary lavage preparations obtained from healthy animals contain a relatively pure population of macrophages (22, 24). Cells obtained by lung lavage were greater than 95% neutral red positive, and Wright’s staining of cytocentrifuged preparations revealed a morphology consistent with being greater than 95% macrophages. The remaining 5% consisted primarily of lymphocytes, and neutrophils were rarely seen. Lymphocytes can also be a source of a cytotoxin (lymphotoxin) (25); however, lymphotoxin is not normally considered to be produced with a LPS stimulus. To determine in our system if cytotoxin production could be accounted for by “contaminating” lymphocytes, adherence studies were done. Table 1 shows the results of two experiments in which nonfractionated and adherent lavage cells obtained from the same animals were tested for cytotoxin production. In both experiments, the contribution of nonadherent cells to cytotoxin production was negligible, indicating that the cytotoxin produced was from adherent cells. Such experiments suggest that macrophages and not neutrophils or lymphocytes were the source of cytotoxin production in our system. Because of the consistent

²The abbreviations used are: LPS, lipopolysaccharide; HEPES, 4-(2-hydroxyethyl)piperazineethanesulfonic acid; MEM, minimal essential medium.
CIGARETTE-IMPAIRED MACROPHAGE TUMORICIDAL ACTIVITY

Table 1

Production of cytotoxin by nonfractionated and adherent pulmonary lavage cells

Pulmonary lavage cells, 4 × 10^6 in 1 ml of medium, were seeded into 24-well plates and incubated for 2 h at 37°C in a 5% CO₂ atmosphere. For adherent cultures, supernatants were aspirated, and adherent cells were washed twice with 1 ml aliquots of medium. After washing, 1 ml of medium was added to the remaining adherent cells. All cultures then received LPS (1 μg/ml). After further incubation for 24 h, supernatants were collected, spun free of cells, and frozen at -20°C until assayed for cytolytic activity. Results for each experiment represent the average of four rats; bars, SD.

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonfractionated lavage</td>
<td>2.6 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adherent lavage cells</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.2</td>
</tr>
</tbody>
</table>

*Mean ± SD.

Chart 1. Kinetics of rat pulmonary lavage cell cytotoxin production. Pulmonary lavage cells, 4 × 10^6 in 1 ml of medium, were seeded into 24-well plates and stimulated with 1 μg of LPS per ml. Cultures were incubated at 37°C in a 5% CO₂ atmosphere, and at shown times, supernatants were collected, spun free of cells, and frozen at -20°C until assayed for cytotoxin activity. Points, average titer from four rats; bars, SD.

homogeneity of our cell preparations and the finding that non-adherent cells contributed negligible amounts of cytotoxin, further studies used nonfractionated cells for the sake of technical simplicity.

Kinetics of Cytotoxin Production. Kinetic studies in the rabbit system have revealed that pulmonary macrophages release maximal cytotoxin titers within 5 to 10 h of LPS stimulation (15). Similar studies were undertaken by us using the rat lavage cells to determine when optimal titers of cytotoxin could be obtained. Chart 1 shows the results of these experiments. Cytotoxin titers became maximal at 10 h after LPS stimulation, and production then plateaued. Over the remainder of the 48-h interval examined, no further increases or diminution of cytotoxin activity occurred. A collection time of 24 h was used for all studies, since this time interval insured maximal production and provided a convenient time for collection of lavage cell supernatants.

LPS Dose-Response of Cytotoxin Production. Chart 2 examines cytotoxin production by lung lavage cells at various doses of LPS. Stimulation became maximal at LPS (0.1 μg/ml), since no further increases in cytotoxin titer could be detected at 1 or 10 μg of LPS per ml. In multiple experiments looking at cytotoxin production by macrophages in the absence of LPS, cytotoxin could not be detected at a supernatant dilution of 1:4 (0.6 log₁₀ units). Thus it appears that some level of LPS is needed for cytotoxin production. Additionally, doses of LPS as high as 100 μg/ml for 24 h did not appear toxic to macrophages as determined by trypan blue staining.

Impairment of Lavage Cell Cytotoxin Production by Cigarette Smoke-exposed Rats

In vivo exposures of rats to cigarette smoke for 8 wk have been shown to produce abnormalities in alveolar macrophage phagolysosome fusion (8). We similarly used this exposure time to examine whether cytotoxin production by lung macrophages was impaired. Table 2 shows the results of four independent experiments. In all experiments, lung macrophages from smoke-exposed rats were significantly impaired in their ability to produce cytotoxin. Differences between cigarette smoke and sham-treated controls varied from 0.7 to 1.0 logs (5- to 10-fold). Thus smoke-exposed lavage cells produced as little as 10% of the levels produced by controls. In several experiments using doses of LPS as high as 100 μg/ml, differences between smokers and controls did not change, suggesting smoke-exposed rats were not simply refractory to the effects of LPS (data not shown).

Impaired Direct Tumor Cytotoxicity by Macrophages from Cigarette Smoke-exposed Rats

To further investigate this effect by more conventional means, we examined the ability of alveolar macrophages from smoke- and air-exposed rats to effect direct tumor cell killing. Chart 3...
CIGARETTE-IMPAIRED MACROPHAGE TUMORICIDAL ACTIVITY

shows the results of such experiments. At macrophage:target cell ratios of 1:1, almost complete killing of targets is seen with little difference between smoke- and air-exposed rats. However, when macrophage:target ratios were decreased, a marked difference appeared in the ability of smoke- and air-exposed macrophages to effect direct tumor cell killing. To effect 50% cytotoxicity, approximately 5-fold more macrophages were required from cigarette smoke-exposed rats than from sham-exposed controls. Thus macrophages of smoke-exposed rats were also impaired in their ability to effect direct tumor cell killing.

Impairment of Interferon Production by Cigarette Smoke-exposed Rats

Interferon, another secretory product of macrophages, is also released with a LPS stimulus. We investigated whether its production was also impaired by chronic smoke exposure. Table 3 shows the results of such an experiment. Interferon production by macrophages from cigarette smoke-exposed rats was markedly decreased (at least by 5-fold) when compared to sham-exposed controls.

DISCUSSION

Cigarette smoking has clearly been implicated as the major cause of lung cancer (26). The current belief is that tumor promoters and carcinogens in tobacco smoke accumulate locally, eventually resulting in neoplastic transformation of respiratory tissue. This concept is supported by a variety of data which have shown that tobacco-derived carcinogens either alone or in the presence of tobacco smoke cause tumors in rats (27), mice (28, 29), and hamsters (30–32), to mention a few studies. With cigarette smoke exposure, impaired macrophage tumor cytotoxicity production may contribute to tumor formation. Decreased tumoricidal capacity of the pulmonary macrophage may impair tumor surveillance and immunity. Similar studies in humans have yet to be carried out. In one published report (11) using human pulmonary macrophages directly as effectors, there was no significant difference in direct cytotoxicity between smokers and nonsmokers against human tumor cells. These investigators, however, used high effector:target ratios and at similar ratios also demonstrated significant toxicity towards normal human fibroblasts. Had the researchers decreased their effector:target cell ratio, considerable differences may have become apparent between smokers and nonsmokers. In another study (12), human pulmonary macrophages were used as effector cells against a human adenocarcinoma cell line target. In that study, effector cell:target cell ratios were varied (0.5:1 to 20:1), and the authors found no significant difference between macrophage cytotoxicity from patients with a diagnosis of bronchial carcinoma and those without carcinoma. The authors, however, did not specifically address differences between smokers and nonsmokers. Our method measures cytotoxin production from large populations of pulmonary macrophages incubated with LPS. Thus, titers are an index of the average amount of cytotoxin released per macrophage. This eliminates the need to use macrophages directly as effectors, and making serial dilutions of cytotoxin is analogous to varying effector:target cell ratios. When we used direct cytotoxicity assays against tumor targets, there was excellent correlation with results obtained by measuring cytotoxin production alone. This effect became particularly apparent when limiting numbers of macrophages were used. Another advantage is the use of units of activity. By using 50% cytotoxicity as a unit, this places titers in the linear portion of the dose-response curve, and respective comparisons between treatment groups can be made. When macrophages as effectors are used, too high an effector:target cell ratio places the comparison in a flat portion of the dose-response curve, disallowing the observation of important differences.

Rabbit studies have shown that maximal cytotoxin release from pulmonary macrophages occurs at levels of LPS (0.1 μg/ml) (15). This is consistent with our studies, since we obtained no higher levels of cytotoxin at LPS doses above 0.1 μg/ml. It is unlikely that smoke-exposed macrophages are less sensitive to LPS stimulation, since a 1000-fold increase in LPS dose did not restore the production to control levels.

The effect(s) of cigarette smoking on secretory products of alveolar macrophages is complex, but increases in the release of superoxide anion (O₂⁻) in humans and in hamsters (24, 33), hydrogen peroxide in rats (34), and elastase and lysozyme in humans (35, 36) all have been observed. Other investigators have shown a decrease in the release of certain arachidonate metabolites in alveolar macrophages from human smokers (37). Similar to the latter situation, our studies show that macrophage tumor cytotoxic release is decreased. Additionally, when we examined interferon, another macrophage product, production was also impaired. These various findings suggest that cigarette smoke constituent(s) may act specifically in their ability to selectively and simultaneously stimulate or depress certain macrophage functions. Interestingly, interferon is known to coactivate (prime) macrophages along with an LPS stimulus to effect tumor

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats used</th>
<th>Interferon titer (log₁₀ units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoke exposed</td>
<td>2</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>Air exposed</td>
<td>4</td>
<td>1.5 ± 0.1*</td>
</tr>
</tbody>
</table>

* Mean ± SD.
cell killing (38). This is evidenced by the need for less LPS in the presence of γ-interferon to effect direct tumor cell cytotoxicity (38) and by the demonstration of increased cytotoxic titers after γ-interferon-containing lymphokine treatment (39). In our system, however, interferon and cytokine productions, although coincident, are probably causally independent events. Macrophage interferon production after LPS stimulation has almost identical kinetics to that found by us for cytoktoxin production (40). To ascribe an enhancing or "priming" effect of interferon on macrophage cytoktoxin production in our system, one would have to postulate that interferon production largely anteceded cytoktoxin production. γ-Interferon is primarily a lymphocyte-derived product, whereas α- and β-interfons are macrophage secretory products and are known to be 500- to 1000-fold less active than γ-interferon in coactivating macrophages (41). In view of this, it is unlikely that the amounts of α- and β-interfons, which would "autoenhance" macrophage cytoktoxin production, could account for the large differences in cytoktoxin titers which we observed between smokers and nonsmokers in our system.

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REFERENCES

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