Cytotoxic Activity of Human Pulmonary Alveolar Macrophages


Departments of Medicine [R. A. K., A. S. B., M. S. M.] and Microbiology [J. K-M., J. C. D. H., M. S. M.], University of Southern California School of Medicine; the University of Southern California Comprehensive Cancer Center [J. K-M., J. C. D. H., R. A. K., V. L. K., M. S. M.], and California Hospital [R. K. R., S. M. S.], Los Angeles, California 90033

ABSTRACT

The functions of human pulmonary alveolar macrophages (PAMs) have been relatively little studied compared with those of their circulating counterparts, blood monocytes. This study examined the ability of human PAMs to kill primary human tumor cell cultures and control normal fibroblasts in vitro. PAMs were derived by bronchial lavage from patients with lung cancer of various histological types and stages, patients with acute or chronic noncancerous pulmonary disorders, and subjects with a presumed illness who proved to be normal. After extensive washing, the PAMs were cocultured with [3H]proline-labeled tumor cells, principally lung cancers and melanomas, at various effector:target ratios for 60 hr. Cytotoxicity was measured by comparing radioactivity associated with the remaining adherent tumor cells cultured in the presence or absence of PAMs. Twenty-eight of 42 preparations of PAMs from 42 individuals were cytotoxic to one or more short-term primary tumor cultures. All 28 specimens from patients with lung cancer or chronic pulmonary disease were cytotoxic; all of the 14 PAM preparations lacking cytotoxicity were from individuals with acute pulmonary disorders or who proved free of pulmonary disease. PAMs were cytotoxic at effector:target ratios of 2.5:1 or 1.25:1. Fibroblasts were unaffected at any ratio. Sarcoidosis patients in remission had noncytotoxic PAMs, whereas the disease in relapse was characterized by cytotoxic PAMs. Serial study of 2 patients confirmed a loss of reactivity during remission. Smoking did not correlate with the presence or absence of spontaneous cytotoxicity and did not influence the degree of cytotoxicity in "reactors." Partially purified α-interferon enhanced the killing of cytotoxic PAMs in 10 of 21 instances but did not induce cytotoxicity in 9 tests on nonreactive PAMs. We conclude that human PAMs from patients with lung cancer or chronic pulmonary diseases, including active sarcoidosis, were cytotoxic to several recently explanted tumor cell cultures. PAMs from acute pulmonary dysfunctions and those from patients with inactive sarcoidosis were not spontaneously cytotoxic.

INTRODUCTION

The macrophage is now recognized as a pivotal cell in both humoral and cell-mediated immunity. In the local and regional defense against foreign substances, microorganisms, or cancer cells, tissue macrophages may have a crucial role, since it is frequently the macrophages that first encounter and must react against the invader. Various functions of peripheral blood monocytes have been studied in normal subjects and many types of patients with chronic conditions, including cancer. Yet the functional state of tissue macrophages is not necessarily the same as that of their circulating counterparts. While the derivation of PAMs is from bone marrow precursors via peripheral blood monocytes, there, appears to be further replication (7, 9) and undoubtedly further differentiation in situ. In order to understand the local response to a cancer or a pulmonary inflammatory stimulus, it is imperative to study PAMs. Relatively little is known about human PAMs however because of their relative inaccessibility compared with blood monocytes. What information exists contains conflicting findings. For example, PAMs from 15 normal individuals were found not to be cytotoxic to SV40-transformed mKSA-TU5 mouse kidney cells, even after stimulation with IFN (2). In contrast, normal PAMs were reported to be spontaneously cytotoxic against 3 long-term human tumor cell lines (12). PAMs can mediate antibody-dependent cell-mediated cytotoxicity to RBC, in fact more efficiently than do bone monocytes because of a higher concentration of Fc receptors on their surface (20). The accessory functions of human PAMs have also been explored to some degree (9, 10) with evidence predominantly for helper cell activity, similar to findings with PAMs in the mouse (8). However, PAMs from the rat and rabbit appear to act principally as suppressor cells (8).

We have studied PAMs obtained by bronchial lavage from patients with acute and chronic pulmonary diseases, including several histological types of bronchogenic carcinoma, and from individuals who proved to be free of significant pulmonary pathology. In this report, we will describe principally the cytotoxic function of PAMs against freshly explanted tumor cells as it is differentially expressed in the several categories of subjects that we studied.

MATERIALS AND METHODS

Subjects. A total of 42 individuals who were scheduled to undergo a diagnostic bronchoscopy agreed to having a bronchial lavage for this study. A consent form approved by the Human Research Committee was signed by each. Subjects were grouped retrospectively into various categories depending upon the final cytological and/or histological diagnosis. The PAMs were tested without knowledge of the final diagnosis, which sometimes took weeks to establish. The specific categories are discussed in subsections of "Results." A complete history, including a careful smoking history, was done by the physicians caring for each patient, as established by our protocol. None of the patients was taking cytotoxic drugs at the time of the study, but 6 of 8 patients with active sarcoidosis were on high doses of corticosteroids.

1 The abbreviations used are: PAM, pulmonary alveolar macrophage; IFN, interferon; E:T, effector to target cell ratio; LPS, lipopolysaccharide; MAF, macrophage-activating factor.
The adenocarcinoma cultures were the most difficult to maintain, tending to die out by the eighth passage. Both the melanoma and squamous carcinoma cultures grew consistently regardless of the number of passages. In these samples, there were fewer than 10% lymphocytes and fewer than 3% contaminating granulocytes. In this study, only 2 samples from sarcomatoid patients had more than 10% lymphocytes. These specimens were discarded for this reason and because the total number of macrophages obtained was too small to permit adequate replicate testing.

Target cells. Primary tumor cultures were established from 3 lung adenocarcinomas, 2 lung epidermoid carcinomas, and 1 melanoma in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum and were purposely cultured continuously for no more than 20 passages or 10 weeks; many were used within 20 passages or 5 weeks. This was done to avoid changes in antigenicity with prolonged cultivation in vitro. The adenocarcinoma cultures were the most difficult to maintain, tending to die out by the eighth passage. Both the melanoma and squamous carcinoma cultures grew consistently regardless of the number of passages. Two human foreskin fibroblast cultures were also established as controls. The identity of cultured cells as tumors was established morphologically by a modified Wright’s stain and functionally by active pinocytosis of neutral red dye, which was used in our studies. A total of 2 to 20 x 10^6 PAMs was obtained from each lavage. Yields from nonsmokers have been at the lower end of this range, while heavy smokers and lung cancer patients have had higher numbers of macrophages in their alveolar spaces. In these samples, there were fewer than 10% lymphocytes and fewer than 3% contaminating granulocytes. In this study, only 2 samples from sarcomatoid patients had more than 10% lymphocytes. These specimens were discarded for this reason and because the total number of macrophages obtained was too small to permit adequate replicate testing.

Reagents. RPMI 1640 or minimal essential medium were purchased in powder form from Grand Island Biological Company (Grand Island, NY) and reconstituted with twice glass-distilled water. Aseptically collected fetal calf serum was purchased from Armour Pharmaceutical Company (Phoenix, AZ). The culture media supplemented with sodium pyruvate, penicillin, streptomycin, and 10% heat-inactivated fetal calf serum were purchased from GIBCO Laboratories, Walkersville, MD.

Partially purified human leukocyte interferon was obtained from the Israel Institute for Biological Research and had a specific activity of 9 x 10^6 U/mg of protein. Its potency was 5 x 10^6 U/mg. This activity was reconfirmed by a viral plaque inhibition assay.

Cytotoxicity Assay. Cytotoxic activity was assessed by measuring [3H]proline retention in prelabeled target cells over a period of 60 hr. Since proline is incorporated into the cytoskeleton and is not secreted directly as scintillation minivials. We compared the radioactivity of target cells cultured in the presence (a) of PAMs with that of cells cultured in the absence (b) of PAMs. Percentage of cytotoxicity was calculated by the formula

\[ \text{% of cytotoxicity} = (1 - \frac{a}{b}) \times 100 \]

In several experiments, we assessed the number of viable PAMs remaining after the incubation by neutral red dye ingestion. By this method, 90 to 100% of the PAMs were found to be viable.

RESULTS

Spontaneous Cytotoxicity Mediated by Human PAMs. Twenty-eight of 42 preparations of PAMs were cytotoxic to one or more of the short-term tumor cell cultures. All of these positive specimens were from patients with lung cancer or a chronic pulmonary disorder. Expressed somewhat differently, 28 of 28 specimens from patients with cancer or chronic pulmonary conditions were cytotoxic to the tumor cell cultures. Otherwise, all of the 14 PAM preparations without cytotoxic activity were from individuals who had acute pulmonary disorders or were proved free of pulmonary disease by the bronchoscopy and other diagnostic procedures.

In preliminary experiments, 8 PAM preparations were tested against 2 melanoma short-term cultures, 2 squamous cell carcinomas, and 3 adenocarcinomas of the lung. All cultures were killed to some degree, but the most consistent killing was found with one melanoma [51 ± 17.7% (S.D.);] and one epidermoid carcinoma culture [51 ± 19.9% (S.D.)]. This spontaneous killing was clearly nonspecific since both lung cancer and melanoma cells were attacked by the cytotoxic PAMs. Adenocarcinoma cells were as sensitive to killing in early passages [51 ± 28.0% (S.D.)] but became less sensitive after they were passaged several times. In fact, these cells appeared to become nesenescent rapidly in culture. Although the epidermoid carcinoma and melanoma were similar in their sensitivity, the ease of cultivation and consistent growth rate of the melanoma made it our principal target cell, used in most of the experiments we will report here.

The effects on the level of spontaneous cytotoxicity of varying the E:T ratio are shown in Chart 1. Four positive specimens were investigated at several different E:T ratios against melanoma and diploid fibroblast cultures. A gradual decrease in cytotoxicity was found as the E:T ratio was decreased from 10:1, which was sufficient to kill 50 to 70% of the targets, to 1:25:1, at which 20% killing above background was still demonstrable. Even at the low E:T ratio of 2:5:1 substantial cytotoxicity of approximately 35% was routinely achieved. This makes it unlikely that contaminating lymphocytes were contributing to these results. Although all types of tumor cells tested were killed, there was some element of selectivity, since the PAMs killed relatively few fibroblasts. There was a mean of 12% cytotoxicity against fibroblasts, contrasted with 50% against melanoma at a ratio of 10:1. We did not have a “standard” cell preparation that could routinely kill fibroblasts to test whether there was some innate resistance of fibroblasts to cytotoxicity, but a similar sparing of adult or late-passage embryo fibroblasts by activated macrophages has long been known in the mouse (6) and has been noted recently by others with human PAMs (19).

Spontaneous Cytotoxicity by PAMs from Lung Cancer and Patients with Chronic Pulmonary Diseases. Spontaneous killing by PAMs obtained from 12 patients with 4 different histological types of lung cancer is illustrated in Table 1. Again the target cell culture shown here was a melanoma. The E:T ratio for all
one with a chronic interstitial pneumonia associated with mixed connective tissue disease; and one with chronic bronchiectasis. Treatment for these patients comprised corticosteroids for 3 of them (with interstitial fibrosis, bronchitis obliterans, and chronic interstitial pneumonia), with such miscellaneous agents as antibiotics (2), bronchodilators (1), Diamox (1), and diuretics (1) also being used. One patient with bronchiectasis was receiving no treatment when the specimen was taken. These individuals had PAMs that mediated a mean of 49% cytotoxicity of melanoma cells, at an E:T ratio of 10:1, with proportionately high cytotoxicity at the other E:T ratios tested (Table 2). Eight patients with active sarcoidosis, i.e., with lesions and symptoms from their disease, had PAMs that caused 35% cytotoxicity at an E:T ratio of 10:1 against melanoma and lung cancer targets. Interestingly, most of these patients (6 of 8) were on high doses of corticosteroids, 60 mg or more per day, at the time their PAMs were tested.

Absence of Spontaneous Cytotoxicity with PAMs from Other Subjects. In direct contrast to the results just described, PAMs from 6 individuals with acute pulmonary conditions, comprising 2 with acute pneumonia, 2 with acute bronchitis, one with dyspnea from sputum exposure, and one with an episode of hemoptysis of unknown cause, failed to cause killing of target melanoma cells at any E:T ratio (Table 3). A mean of -3.5% cytotoxicity ±6.5% (S.E.) was noted. We also had the opportunity to study 8 patients with sarcoidosis during a remission from their disease. The mean cytotoxicity for these 8 patients was 2.1 ± 6.3% (S.E.). Two of the sarcoidosis patients were studied serially, both of whom had had PAMs that were cytotoxic during the active phase of their disease (45 and 37%). Three PAM preparations obtained during remission from the 2 patients failed to kill target tumor cells significantly, 10, 11, and -21% against melanoma at an E:T ratio of 10:1. Thus, a reversion to the normal pattern of nonreactivity occurred with a change in clinical status towards normal. We have not yet had the opportunity to study a patient with lung cancer put into remission by treatment to test for any similar changes in PAM reactivity. Note that normal individuals (volunteers) were not studied for comparison because of ethical constraints.

Influence of Smoking on Cytotoxicity. An obvious question that occurred to us was whether cigarette smoking was a major determinant of cytotoxicity of the PAMs, as a stimulator or

tests shown here was 10:1, except for Patient A. B., where it was 7:5:1.

All 12 patients with bronchogenic carcinoma had PAMs that were spontaneously cytotoxic against one or more primary lung or melanoma tumor cell cultures, with relatively little reactivity against normal foreskin fibroblasts. While we tested only a limited number of patients in each subgroup, PAMs from 8 patients with Stage I disease appear to be less cytotoxic than do those derived from 4 patients with more advanced Stage II and III disease, killing melanoma targets at 39% as opposed to 74% (p = 0.002 by the Mann-Whitney U test).

The other patients with consistently cytotoxic PAMs were six with "chronic pulmonary disease." Included in this category were the following: 2 individuals with chronic nonspecific bronchitis; one with idiopathic bronchitis obliterans accompanied by interstitial fibrosis; one with interstitial fibrosis of unknown etiology;
perhaps an adverse influence. Data in Table 4 analyzing 42 patients indicate that no direct relationship was present between smoking and spontaneous cytotoxicity of PAMs. Eleven of 28 patients whose PAMs were cytotoxic were nonsmokers. More significantly perhaps, 8 of the 14 noncytotoxic macrophage preparations came from moderate to heavy smokers. By \( \chi^2 \) analysis, with Yates’ correction for small groups, there were no significant differences between the groups, \( (\chi^2 = 0.0405; p = 0.841) \).

The degree of cytotoxicity was similar for PAMs from cancer patients who smoked and those who did not (i.e., those who had given up smoking more than 2 years before the test). The comparable figures for these groups for cytotoxicity were 55 ± 7.1% (S.E.) and 41.5 ± 6.5% (S.E.). Among the noncytotoxic PAMs there was a uniform failure of cells from smokers and nonsmokers alike to mediate killing, with a mean of 5% for each and no more than 16% cytotoxicity within either subgroup. These data fail to substantiate a significant influence of smoking per se on killing by PAMs.

**Effect of IFN.** Extracted, partially purified leukocyte IFN-α was added to mixtures of PAMs and target tumor cells at the beginning of the 60-hr incubation period. When tested with PAMs from patients with lung cancer, active sarcoidosis, or other active chronic pulmonary diseases, IFN-α increased cytotoxicity in 10 of 21 tests (Table 5). PAMs from lung cancer patients were most consistently increased in reactivity (5 of 6 experiments on 6 patients), with the least consistent increases found in patients with active sarcoidosis (2 of 8 tests on 8 patients). The degree of augmentation was variable, ranging from an 8% absolute increase to a doubling of reactivity, but the mean absolute increase was 16%, (representing a relative change of approximately 50%) for 2 of the 3 active groups. In sarcoidosis patients, not only was there a less frequent augmentation of PAMs by IFN, but there was also a smaller mean increase (6%) in those who responded. In direct contrast, extracted IFN-α did not stimulate any of the 9 specimens of PAMs from individuals with acute pulmonary conditions or inactive sarcoidosis to become cytotoxic, even at E:T ratios of 10:1 or higher (Table 5).

<table>
<thead>
<tr>
<th>No. of smokers</th>
<th>% of cytotoxicity</th>
<th>No. of non-smokers</th>
<th>% of cytotoxicity</th>
<th>Total no. of subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytotoxic</td>
<td>17 (45.0 ± 5.0)</td>
<td>11 (44.0 ± 6.9)</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Noncytotoxic</td>
<td>28 (5.2 ± 8.3)</td>
<td>17 (4.8 ± 6.5)</td>
<td>45</td>
<td></td>
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\( \chi^2 = 0.0405 \)

\( p = 0.841 \)

*Mean ± S.E. at an E:T ratio of 10:1 against melanoma cells.*

These studies indicate that normal human PAMs are not spontaneously cytotoxic to recently explanted tumor cells, whereas PAMs from patients with lung cancer or a chronic pulmonary disease are. Stated somewhat differently, PAMs are activated to become nonspecifically cytotoxic by the presence of a “chronic” local process, such as lung carcinoma or sarcoidosis (and, interestingly, not by acute processes such as pneumonia), rather than having innate cytotoxic properties. These results with human short-term cultures of tumor cells as targets are consistent with the observations of Bordignon et al. (2) who found no cytotoxicity against an SV40-transformed mouse cell line, mKSA-TU 5, with 15 preparations of PAMs from normal human donors. Lemarbre et al. (12) found that all human PAMs tested, whether from normal subjects or cancer patients, were cytotoxic, with a “trend” towards higher cytotoxicity with the latter. However, their target cells were long-term cell lines that may have been too sensitive to cytotoxicity to discriminate between populations of actual and potential killer cells. The importance of using only short-term human tumor cell cultures as targets for cytotoxic reactions to obtain specificity has been emphasized by several groups, including our own, in the study of human peripheral blood mononuclear cell-mediated cytotoxicity against melanoma or ovarian carcinoma (4, 15, 16). The absence of spontaneous cytotoxicity with normal PAMs in vitro does not vitiate the suggestion that PAMs are important in local surveillance against lung cancer cells. It would suggest, however, that other influences, such as mediators from lymphocytes, must also play a role if the macrophages are to be activated rapidly enough to recognize and destroy the foreign cells before they can threaten the patient.

Smoking did not appear to influence in either direction the ability of PAMs to kill tumor cells. Certainly smoking did not destroy or diminish the ability of PAMs from lung cancer patients to kill, and cytotoxic PAMs were found in the same proportion of nonsmokers as in the smokers (one-third). On the other hand, cytotoxic PAMs were found in nonsmokers too (8 of 14) in almost exactly the same proportion as in the smokers (17 of 28), so that smoking per se did not cause the cytotoxicity we measured. A major influence of bacterial LPS (endotoxin) on cytotoxicity was unlikely. Activation of human blood monocytes requires
normal PAMs were not made cytotoxic by IFN or MAF-containing whereas it did augment the cytotoxicity of PAMs that already continually being replaced by a new population of noncytotoxic in the absence of activation stimuli, or because the population is either because the individual PAM reverts to noncytotoxic in blood (1) or by replication in situ (9).

Several groups have reported that circulating human monocytes from normal individuals as well as from lung cancer patients can kill or inhibit the growth of lung carcinoma cells (21) and other murine and human tumor cell lines (5, 13) but generally cannot kill or inhibit the growth of fibroblasts or phytohemagglutinin-transformed lymphocytes (5, 14). There was no correlation of cytostatic activity of peripheral blood monocytes with clinical stage (21), nor was there a significant difference in cytostatic or cytotoxic activity between normal and various types of cancer patients, including those with lung cancer (5, 21). These findings differ considerably from ours, perhaps in part because cell lines or virally transfomed target cells were used in those studies, but also because normal blood monocytes may be spontaneously cytotoxic in contrast to PAMs. The reported degree of augmentation of cytotoxicity of blood monocytes by lymphokines has varied from minimal (5) to significant (2); lymphokines induced only weak cytotoxicity in normal PAMs (4). Our data on 12 patients thus far suggest a correlation between the stage of the patient’s lung carcinoma and the degree of reactivity of PAMs (Table 1). It is somewhat discomfoting that the higher degrees of killing were found in patients with more tumor burden rather than those with less, although the groups require further expansion. Taken as a whole, our data from this study suggest that by developing cytotoxicity, the PAMs may simply have been reacting to the presence of the tumor but were not protecting against its occurrence. The change of nonreactivity in normal individuals to reactivity in the presence of cancer may be a matter of the number of macrophages activated by the disease process, and hence the degree of lysis produced at various E:T ratios. Whether there are quantitative changes in cytotoxicity differing with each stage of the cancer due either to the length of time the tumor was present or the tumor burden will receive further scrutiny. In any event, if the degree of reactivity of PAMs were to contribute to more accurate staging of lung cancer, a significant benefit could accrue to the patient through more definitive management. The reversion of PAMs from sarcoidosis patients to the noncytotoxic state with remission of the disease is a finding that strongly supports the concept that continuous stimulation is required to elicit continued cytotoxic activation, either because the individual PAM reverts to noncytotoxicity in the absence of activation stimuli, or because the population is continually being replaced by a new population of noncytotoxic macrophages either from the bone marrow via the peripheral blood (1) or by replication in situ (9).

IFN alone did not cause noncytotoxic PAMs from normal individuals or sarcoidosis patients to kill target tumor cells, whereas it did augment the cytotoxicity of PAMs that already had some capacity for killing. Bordignon et al. (2) found that normal PAMs were not made cytotoxic by IFN or MAF-containing supernatant fluid, whereas peripheral llood mononuclear cells were stimulated by MAF. The IFN-containing material we used for our studies as "IFN" was impure and undoubtedly contained a variety of leukocyte-made substances. Nevertheless, even this mixture failed to stimulate the noncytotoxic PAMs. It is still probable, now that we know that human PAMs are in fact capable of becoming cytotoxic, that several influences brought to bear on the macrophage in sequence could enable IFN to activate the PAM. The increased killing capacity by IFN-stimulated PAMs taken from chronic pulmonary diseases undoubtedly reflects such a sequential stimulation, and the contention is also bolstered by preliminary findings in our laboratory that MAF-containing supernatant fluids from phytohemagglutinin- or concanavalin A-stimulated T-cell cultures can stimulate PAMs to become cytotoxic.6 The spontaneous reactivity of PAMs in active sarcoidosis and the lack of augmentation by IFN in this setting might conceivably be attributed to prior dual stimulation by lymphokines and IFN in vivo.

Since the macrophage subserves a wide variety of roles, whose importance in the prevention of lung cancer or the pathogenesis of chronic inflammatory lung disease is unknown, we have been studying many activities of PAMs concomitantly. Antibody-dependent cell-mediated cytotoxicity which is mediated more effectively by PAMs than by peripheral blood monocytes (20), the production of monokines such as interleukin 1, and the modulatory (helper and suppressor) functions of macrophages may all be of importance in relation to various disease states. Our initial focus on cytotoxicity in some depth is simply a way of beginning to dissect the situation rather than an assumption of its preeminence in the pathogenesis of either lung cancer or chronic pulmonary diseases. In fact, we feel that the process of "activation" of the macrophage has, in many ways, been too vaguely defined to be useful. Several specific attributes of the activated state should be examined to decide whether activation of some sort has been achieved. When interleukin 1 is produced, for instance, morphological activation (e.g., spreading or ruffling of the cell membrane) does not necessarily occur. By the same token, PAMs termed activated by one functional criterion, such as cytotoxicity, may not necessarily be considered activated by another. The type of activation of PAMs, or other macrophages, and the stimuli involved should certainly be specified.

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