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Chemiluminescence, Suppression and Cytotoxicity in Peripheral Blood Mononuclear Cells from Solid Tumor Cancer Patients

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Summary. *Chemiluminescence, indomethacin-sensitive suppression, and adherent cell cytotoxicity were measured in peripheral blood mononuclear cells (PBMC) from normal subjects and solid tumor cancer patients. These functions were found to be differentially affected by malignant disease. In cancer patients with disseminated disease, indomethacin-sensitive suppression and chemiluminescence emission were increased to a level significantly higher than normal without a concurrent increase in adherent cell cytotoxic function. In cancer patients with at most minimum residual diseases, the levels of chemiluminescence, indomethacin-sensitive suppression, and adherent cell cytotoxicity found were comparable to those of the normal study population. In vitro stimulation of cells from patients with disseminated disease by phorbol myristic acetate (PMA) increased chemiluminescence overcame the suppressive effects of indomethacin-sensitive suppressor cells, and increased adherent cell cytotoxicity; in cells from patients with at most minimum residual disease, PMA increased chemiluminescence and cytotoxicity without influencing the activity of indomethacin-sensitive suppressor cells. Vaccination of lung cancer patients with Freund's complete adjuvant or Freund's complete adjuvant plus tumor antigen extracts led to increased levels of chemiluminescence and increased levels of adherent cell cytotoxicity without altering indomethacin-sensitive regulatory cell function.*

Introduction

The influence of human malignant disease on those immune functions mediated or influenced by monocytes, particularly in relationship to stage of disease, prognosis, and responsiveness to therapy is not completely clear [3, 10, 13, 14, 17, 19, 23, 26, 27, 29, 31, 32]. We and others have described the occurence of elevated levels of immunoregulatory suppressor function by adherent peripheral blood cells from solid tumor patients [10, 12, 28, 33]. Modulation of T cell responsiveness by adherent cells has been related to the effects of prostaglandins [7]. We have also observed that cells from solid tumor patients exhibit elevated levels of chemiluminescence emission (CL) following isolation of peripheral blood mononuclear cells (PBMC) and enhanced CL emission following incubation with phorbol myristic acetate (PMA) compared with cells from normal subjects [11]. CL emission is thought to be due to the relaxation of electronically excited carbonyl groups due to oxidation of ingested materials by singlet oxygen [5], and is correlated with monocyte phagocytic activity [25, 30] and may be associated with a production of prostaglandins [37]. Results of studies of adherent cell cytotoxic function in cancer patients have varied; some studies demonstrate depressed cytotoxic function in cancer patients [21], while others demonstrate normal or even increased levels of function in those patients [19].

Then too, the influence of various forms of immunotherapy on monocyte-related functions of human cancer patients remains largely to be determined. It might be expected that successful immunotherapy would augment monocyte cytocidal functions without stimulating monocyte suppressor functions. Yet some studies suggest that each function is augmented in cancer patients who are treated with BCG [19].

In the present study, we have simultaneously measured levels of CL emission, indomethacin-sensitive immunoregulatory function, and adherent cell cytotoxic function in PBMC from solid tumor cancer patients. Modulation of these functions in vitro by PMA and in vivo by immunotherapy was also investigated.

Materials and Methods

Patient Population. The patient population consisted of 27 patients with non-lymphoreticular solid tumors, 15 of whom were classified as having disseminated disease (including 6 patients with bronchogenic carcinoma, four patients with colon cancer, two patients with head and neck cancer, one patient with hypernephroma, one patients with bladder cancer and one patient with melanoma) and 12 as having at most minimum residual disease (including 7 patients with stage I or II lung cancer and 5 with stage II breast cancer). The patient population ranged in age from 30 to 70 years (median age, 51 years). For purposes of this study, 'disseminated disease' indicates disease that has spread beyond regional lymph nodes draining the site of the primary tumor. Patients with disseminated disease were studied a minimum of 1 month following surgical biopsy or operation and prior to initiation of radiation or chemotherapy. Patients with at most minimum residual disease were studied 10–30 days following definitive surgical resection of disease. They were studied prior to beginning adjuvant programs of chemotherapy (breast cancer patients) or adjuvant programs of immunotherapy (lung cancer patients). They are referred to in the remaining portions of this paper as patients with 'minimum disease' or 'at most, minimum

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residual disease.' The justification for their inclusion in this study is provided in the *Discussion*. The control population consisted of 15 healthy individuals ranging in age from 21 to 70 years (median age, 35) none of whom was receiving any medication. We also included in this study, lung cancer patients who, following surgical resection of disease, were randomized to receive (i) no further therapy; (ii) 3-monthly injections of Freund's complete adjuvant (FCA); or (iii) 3-monthly injections of FCA emulsified with extracted lung tumor antigens (TAA) [34].

Isolation of PBMC. Whole blood (heparinized at 10 U/ml) was obtained from healthy adult volunteers or patients by venipuncture. The blood was layered on lymphocyte separation medium (LSM, Bionetics, Kensington, MD, USA) and centrifuged at 480 *g* for 20 min at room temperature. Platelets were removed by slow-speed centrifugation. Isolated PBMC were washed twice in Hank's balanced salts solution, and mononuclear phagocytes were enumerated by latex ingestion. Smears were made of isolated PBMC and stained with Wrigth's stain to determine percentages of lymphocytes, monocytes, and other cell types, such as granulocytes in the mononuclear cell preparation. A granulocyte contamination level of 5% or less was seen in PBMC preparations from normal subjects. Thus, individual patients and patient samples were rejected from inclusion in this study when any sample contained 5% or more granulocyte contamination. These criteria were invariably satisfied by all preparations from patients with minimum residual disease and by the majority of preparations from patients with untreated disseminated disease considered for this investigation.

Chemiluminescence Assay. The CL assay was a modification of the procedure described by Allen [3]. Glass scintillation vials containing 1 nmol luminol were dark-adapted for 1 h. PBMC adjusted to contain 1×10^5 mononuclear phagocytes in barbitol buffer were then added in a total volume of 2.0 ml and CL was monitored at ambient temperature for 1–2 h in a Packard scintillation spectrometer in the out-of-coincidence mode. When stimulated CL was measured, only 7×10^3 mononuclear phagocytes/vial were used. Background CL was monitored as described above for 10 min, after which 10 µg PMA was added and ligth emission was monitored for 1–2 h.

Lymphoproliferation Assay. Peripheral blood mononuclear cells from cancer patients were tested for their proliferative response to a 1 : 500 dilution of PHA in 72-h cultures. These culture conditions were found to elicit optimum DNA synthesis in cells from normal individuals and cancer patients. Briefly, PBMC were resuspended in RPMI-1640 medium supplemented with 20% heat-inactivated fetal calf serum (FCS), 100 U penicillin/ml and 100 µg streptomycin/ml to a concentration of 2×10^6/ml, and 100 µl cells was dispensed into triplicate wells of a sterile, 96-well Linbro microtiter plate. To individual wells, 100 µl of a 1 : 500 dilution of a 1% stock solution of PHA (Difco, lot # 649848) was added, and subsequently cultures were incubated for 72 h at 37° C in a humidified atmosphere of 5% CO_2 in air. During the last 6 h of culture, cells were labelled by adding 1 µCi ^{3}H-thymidine to each well, following which cells were harvested with the aid of a multiple automated sample harvester (MASH II) and incorporated radioactivity was measured by liquid scintillation counting. Lymphocyte DNA synthesis is expressed as mean counts per minute (cpm) for triplicate wells of individual cultures (standard deviation of the mean never exceeded 15% of cpm).

Assessment of Indomethacin-Sensitive Immunoregulatory Cells. Washed PBMC were resuspended to a final concentration of 2×10^6/ml in RPMI 1640 medium supplemented with 20% FCS, 100 U penicillin/ml, and 100 µg streptomycin/ml and containing 2 µg indomethacin/ml [35, 36]. Indomethacin (Sigma, St. Louis, USA) was dissolved in absolute ethanol and diluted in RPMI 1640 medium. Control cultures contained the same amount of ethanol as did indomethacin-treated cultures and no influence on resultant PHA responses were seen. Cells were stimulated with PHA for 72 h as described above, following which lymphocyte DNA synthesis was determined. In some instances, PMA (phorobol myristic acetate) (10^{-7} *M*, final concentration) was added to the cell cultures in addition to PHA or indomethacin.

Adherent Cell Cytotoxicity Assay. PBMC were suspended in RPMI 1640 medium containing 10% heat-inactivated FCS, 50 U penicillin/ml, and 50 µg streptomycin/ml and placed in flat-bottom microplates to yield 15×10^4 mononuclear phagocytes/well. These were incubated overnight at 37° C in an atmosphere of 5% CO_2, following which nonadherent cells were gently removed by aspiration. The adherent cell population was washed three times with medium prior to addition of target cells. Target cells were Chang liver cells maintained in RPMI 1640 medium containing 20% FCS, 50 U penicillin/ml and 50 µg streptomycin/ml and passaged weekly. Target cells (1×10^6) were labeled with 50 µCi ^{51}Cr for 1 h at 37° C, following which they were washed three times with medium containing 1% BSA and subsequently resuspended to 1×10^5/ml in RPMI medium containing 10% FCS, 50 U penicillin/ml and 50 µg streptomycin/ml. To wells containing adherent cells, 1×10^4 labeled target cells were added, cultures were incubated for 18 h, following which supernatants were collected and percentage cytotoxicity determined. The percentage specific cytotoxicity was calculated as:

$$\% \text{ Specific cytotoxicity} = \text{E-S} \div \text{m-s} \times 100,$$

where E = cpm released with adherent cells present; S = cpm released spontaneously (without adherent cells present) and M = maximum release determined by target cell lysis with 2% SDS.

Results

Chemiluminescence, Immunoregulatory Function, and Cytotoxicity in PBMC from Solid Tumor Cancer Patients

Chemiluminescence (CL), indomethacin-sensitive immunoregulatory function, and adherent cell cytotoxic function were simultaneously measured in PBMC from solid tumor patients with either advanced disease or at most minimum residual disease and from normal subjects. Levels of immunoregulation by indomethacin-sensitive suppressor cells were expressed as an augmentation index calculated as the mean cpm of PHA-stimulated cultures in the presence of indomethacin divided by the mean cpm of PHA-stimulated cultures in the absence of indomethacin [10]. Patients with advanced disease as a group were found to have significantly elevated levels of

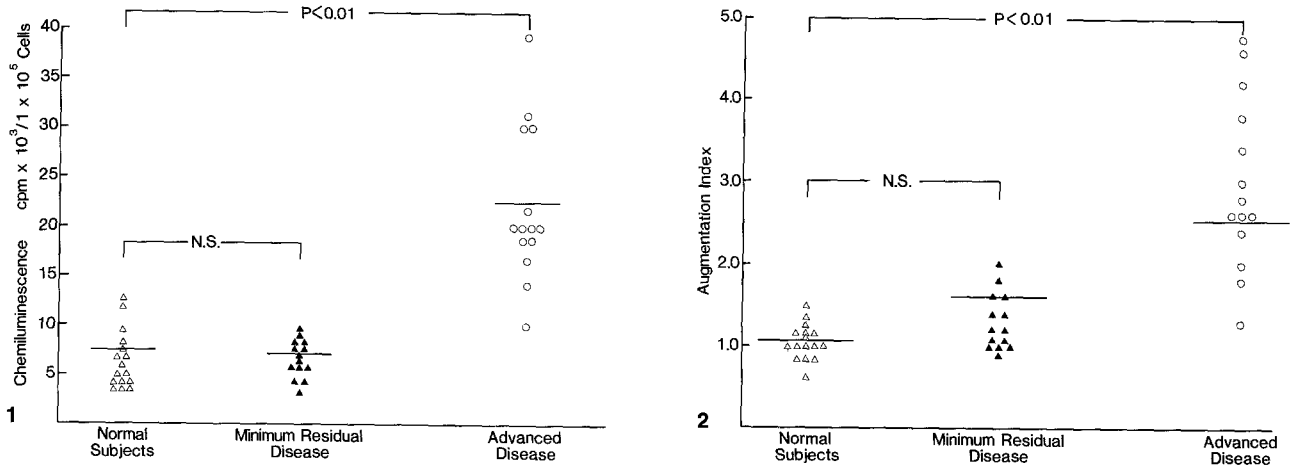


Fig. 1. CL emission from PBMC of normal subjects, cancer patients with at most minimum residual disease, and cancer patients with advanced disease. Statistical comparison of groups was by Wilcoxan rank sum test. NS, not significant

Fig. 2. Indomethacin-induced augmentation of PHA-stimulated DNA synthesis on PMBC from normal subjects, cancer patients with at most minimum residual disease, and cancer patients with advanced disease. Statistical comparison of groups was by Wilcoxon rank sum test. NS, not significant

CL emission and indomethacin-sensitive suppressor function in their PBMC compared with normal subjects ($P < 0.01$ and $P < 0.01$ by Wilcoxon rank sum statistic, respectively) (Fig. 1). The levels of adherent cell cytotoxic function found varied from 1% to 14% specific cytotoxicity, which was comparable to the values found in the normal subject population. Patients with at most minimum residual disease as a group were found to have levels of CL, indomethacin-sensitive suppression, and adherent cell cytotoxicity comparable to those of the normal subject group. The levels of each function were considered to be significantly elevated above normal when an individual value for any function in the patient's PBMC was greater than two standard deviations (SD) above the mean level of function in that test for the normal subject group. In patients with disseminated disease, three patients had significantly elevated CL levels alone, two patients had significantly elevated indomethacin-sensitive suppressor function alone, eight patients had significantly elevated levels of both CL and indomethacin-sensitive suppression, and no patients had significantly elevated levels of adherent cell cytotoxicity. None of the patients with at most minimum residual disease, had significantly elevated levels of CL or adherent cell cytotoxicity, while four patients had significantly elevated levels of indomethacin-sensitive suppression.

Correlation of CL, Indomethacin-Sensitive Suppression, and Cytotoxicity in PBMC from Cancer Patients

The relationship between CL, indomethacin-sensitive suppression, and adherent cell cytotoxicity in PBMC from cancer patients was tested by linear regression analysis. A significant positive correlation was found between CL and indomethacin-sensitive suppressor function for patients with disseminated disease ($r = 0.56$, $P < 0.01$) but not for patients with at most minimum residual disease ($r = 0.21$, not significant). No significant correlation was found for adherent cell cytotoxicity and CL in patients with disseminated disease ($r = 0.32$, NS) or patients with at most minimum residual disease disease ($r = 0.30$, NS); also, no significant correlation was found between adherent cell cytotoxicity and indomethacin-sensitive suppression in patients with disseminated disease ($r = 0.35$, NS) or patients with at most minimum residual disease disease ($r = -0.29$, NS).

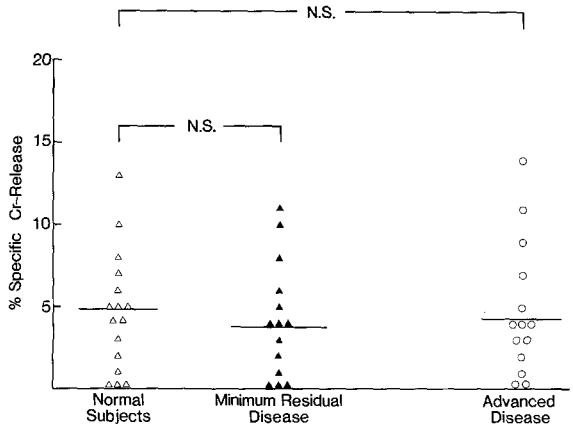
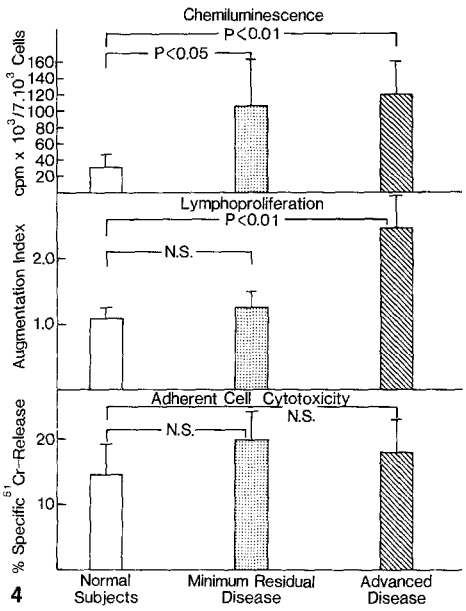


Fig. 3. Cytotoxicity against Chang liver cells by glass-adherent PBMC from normal subjects, cancer patients with at most minimum residual disease, and cancer patients with advanced disease. Statistical comparison of groups was by Wilcoxon rank sum test. NS, not significant

Effects of in vitro Treatment of PBMC with PMA on Levels of CL, PHA, Responsiveness, and Cytotoxicity

Phorbol esters are known to exert multiple effects on the immune system. In many instances, the effects seen have been attributed to the influence of these substances on monocyte function [1, 15]. In the present study, isolated PBMC were assessed for CL, PHA responsiveness, and cytotoxicity in the presence of PMA (Fig. 4). The influence of PMA on PHA responsiveness was also expressed as an augmentation index calculated as the cpm of PHA-stimulated cultures in the presence of PMA divided by the cpm of PHA-stimulated cultures in the absence of PMA. PMA treatment of PBMC significantly enhanced CL in each of the populations studied



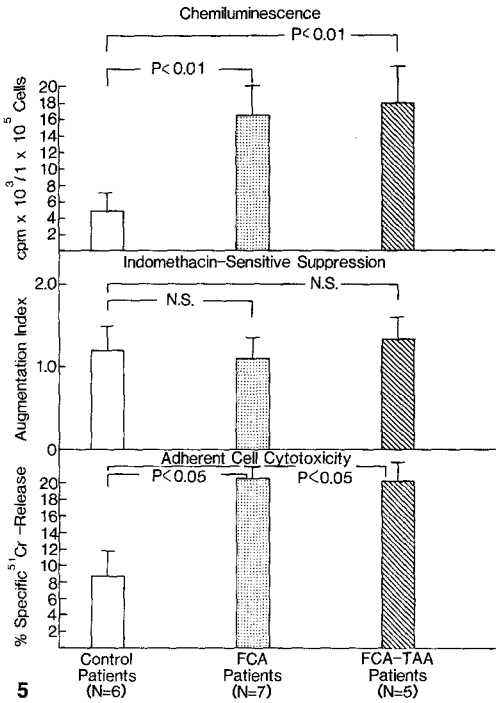


Fig. 4. Effect of PMA on CL, PHA-induced lymphocyte DNA synthesis, and adherent cell cytotoxicity by PBMC from normal subjects, cancer patients with at most minium residual disease, and cancer patients with advanced disease. Statistical comparison of groups was by Wilcoxon rank sum test. NS, not significant.

Fig. 5. Chemiluminescince, indomethacin-sensitive suppression and adherent cell cytotoxicity by PBMC from surgically resected lung cancer patients who received no further therapy (control patients), 3-monthly injections of FCA (FCA patients), or 3-monthly injections of FCA emulsified with extracted lung tumor-associated antigens (FCA-TAA patients). Statistical comparison of groups was by Wilcoxon rank sum test. NS, not significant.

($P < 0.05$ for normal subjects and $P < 0.01$ for patients with minimum disease and disseminated disease). The level of PMA-stimulated CL seen in PBMC from the cancer patient groups was significantly different from the level of PMA-stimulated CL seen in PBMC from normal subjects ($P < 0.05$ and < 0.01 for minimum disease and disseminated disease patients, respectively). PMA treatment of PBMC from normal subjects, minimum disease cancer patients, or advanced disease cancer patients was also found to enhance adherent cell cytotoxic function. In this case, however, the augmentation of cytotoxicity found with PMA was comparable for each study population. PHA-stimulated DNA synthesis by PBMC from normal subjects or minimum disease cancer patients was not significantly altered by PMA (Fig. 4) (mean augmentation indices were 1.1 and 1.2, respectively). However, PHA-stimulated DNA synthesis by PBMC from disseminated cancer patients was significantly augmented in the presence of PMA (mean augmentation index was 2.4).

Effects of Immunotherapy on CL, Indomethacin-sensitive Suppressor Function, and Adherent Cell Cytotoxicity in PBMC from Lung Cancer Patients

The influence of immunotherapy on CL, indomethacin-sensitive suppression and adherent cell cytotoxicity was evaluated in PBMC from surgically resected lung cancer patients who received no further therapy (6 patients), FCA alone (7 patients), or FCA + TAA (5 patients). The results obtained for these patients 6 months following their entry on study and 3 months following their final vaccination are presented here (Fig. 5). PBMC from lung cancer patients who received no further therapy exhibited normal levels of baseline CL, normal levels of indomethacin-sensitive suppressor function, and normal levels of adherent cell cytotoxicity. On the other hand, PBMC from lung cancer patients who were vaccinated with FCA or FCA + TAA exhibited significantly elevated levels of baseline CL ($P < 0.05$ for both groups), normal levels of indomethacin-sensitive suppression, and significantly elevated levels of adherent cell cytotoxicity ($P < 0.05$ for both groups).

Discussion

The results of this study suggest that CL, indomethacin-sensitive suppression, and adherent cell cytotoxicity in PBMC are differentially affected by malignant disease. Indomethacin-sensitive suppression and CL emission were increased in PBMC from cancer patients with disseminated disease without a concurrent increase in adherent cell cytotoxic function. Normal levels of CL and adherent cell cytotoxicity were found in association with elevated levels of indomethacin-sensitive suppression in PBMC from cancer patients with minimum disease. When analyzing individual patients, it was found that significantly increased CL emission coincided with significantly increased indomethacin-sensitive suppression in some but not all patients with disseminated disease, but never in patients with minimum disease. Different effects of immune modulators on these functions were also found in this study. In vitro treatment with PMA increased CL, increased depressed PHA

responsiveness, and increased adherent cell cytotoxicity in PBMC from patients with disseminated disease. The same treatment increased CL and adherent cell cytotoxicity without altering PHA responsiveness in PBMC from minimum disease patients. Vaccination of lung cancer patients with FCA or FCA + TAA also led to increased levels of CL and increased levels of adherent cell cytotoxicity in patient PBMC without altering indomethacin-sensitive regulatory function in these cells.

Each immune function tested in this study is known to be mediated, or at least influenced, by the actions of monocytes. Augmentation of mitogen-induced lymphocyte DNA synthesis in indomethacin-treated leukocyte cultures is thought to result from the inhibition of prostaglandin synthesis and secretion by monocytes. The contribution of such cells to the immunodeficiency of cancer patients has been well documented [18]. Our assessment of suppressor activity employing indomethacin addition to whole PBMC cultures is, of course, indirect. Recently, however, we have demonstrated that increased levels of indomethacin-sensitive suppressor function are directly correlated with increased conversion of arachidonic acid to prostaglandins of the E series on the part of peripheral blood glass-adherent cells [8]. There is a substantial body of evidence which suggests that CL provides a reliable, quantifiable assay of oxidative metabolism in reticuloendothelial cells [2, 4, 5, 6]. Macrophage activation has been assessed using CL [30], and human monocytes also respond with light emission to a variety of stimuli [20, 25]. Although lymphocytes are known to chemiluminesce, the level of light emission detected is at least one order of magnitude below that of monocytes. In our studies, glass-non-adherent cells did not emit detectable CL under the conditions used in this study (data not shown). Nonetheless, we do not exclude the possibility that lymphocyte actions may contribute in a positive or negative way to the CL of PBMC. Studies of adherent cell cytotoxicity in man have demonstrated that a certain proportion of peripheral blood monocytes exist in an 'activated' state and may be further activated by lymphokines, tumor products, or glass adherence [21]. Neither the influence of malignant disease nor the influence of immunotherapy on this function has been well studied in cancer patients.

PMA is known to influence leukocyte functions. For example, PMA is known to induce CL in PMN [30] and monocytes [30]. Increased phagocytosis and prostaglandin synthesis [25, 30, 37] by PMA-treated monocytes has also been reported. That PMA can augment lymphocyte DNA synthesis through a mitogen-like action and augment monocyte cytotoxic functions, perhaps through enhancement of H_2O_2 production, has also been reported [1, 15, 24]. In the present study, treatment of PBMC in vitro with PMA increased chemiluminescence, PHA-induced lymphocyte DNA synthesis, and adherent cell cytotoxicity. The effect of PMA on the functions tested was at least partially dependent upon the source of cells employed in these studies. Thus, PMA elevated CL levels in PBMC from cancer patients to a significantly greater extent than in cells from normal subjects. On the other hand, PMA augmented adherent cell cytotoxicity to a similar extent for all the groups studied.

Some of the patients included in this study might have been disease-free, whereas others would have residual low tumor burdens. Early stage cancer patients are useful to study as a group, for as a group they are entered in adjuvant programs of chemotherapy or immunotherapy. It is important to document their immune status prior to their beginning these programs. Serial immune monitoring thereafter might permit a definition of those changes in immune function that are associated with clinical tumor recurrence.

Vaccination of lung cancer patients with materials containing FCA led to stimulation of chemiluminescence and adherent cell cytotoxicity in patient PBMC without increasing indomethacin-sensitive suppression. Other studies of immunotherapy of lung cancer have suggested that treatment with BCG can lead to stimulation of adherent cell cytotoxicity but at the expense of increasing monocyte suppression [19]. This was taken to indicate that the activated cytotoxic monocyte was also responsible for suppressing lymphoproliferative responses. Then too, activated monocytes may enhance or suppress immune responses depending on the ratio of monocytes to lymphocytes in the test culture. This did not appear to be a problem in our immunotherapy patients, at least in terms of indomethacin-sensitive suppressor cells. Also, recent studies using elutriation centrifugation indicate that cytotoxic monocytes can be separated from suppressor monocytes by size. The nature of the vaccine employed or the treatment schedule utilized may be a critical determinant of the effects of therapy on monocyte-related functions. The biological responses of cancerous hosts to vaccination is known to be dependent upon a multitude of such variables [9, 23]. Whether the therapeutic efficacy of vaccination for stage I and stage II lung cancer is related to modulation of monocyte-associated functions remains to be seen [33].

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