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Chemiluminescence in Peripheral Blood Mononuclear Cells of Solid Tumor Cancer Patients

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Summary. *Levels of chemiluminescence were measured in peripheral blood mononuclear cells (PBMC) from normal subjects and from solid tumor cancer patients. Patients with advanced malignant disease were found to have significantly elevated baseline chemiluminescence activity in their 'resting PBMC' as compared to normal subjects or to cancer patients with, at most, minimum residual disease. Patients with either advanced disease or minimum residual disease, however, were found to exhibit significantly elevated activation of chemiluminescence by treatment of cells with phorbol myristic acetate (PMA). Treatment of surgically resected stage I lung cancer patients with Freund's complete adjuvant alone or emulsified with extracted lung cancer antigens was found to elevate chemiluminescence levels in patient PBMC. Serum from those vaccinated patients was found to elevate chemiluminescence levels of resting PBMC from normal subjects. That serum activity did net correlate with levels of immune complexes measurable in the C1q or Raji cell assay.*

Introduction

Cancer is known to affect the functional status of host monocytes and macrophages [27]. The nature and extent of the changes produced appear to be correlated with tumor burden [18, 24, 27] and are thought to reflect both host antitumor reactions and the influences of tumor-derived factors [18, 24]. Some of the functional changes described include increased immunoregulatory suppressor activity [8, 21], enhanced cytotoxic function [15], enhanced phagocytic capabilities [18], decreased response to chemotactic stimuli [18, 26] and decreased cutaneous inflammatory activity [9, 26].

The metabolic conditions which characterize the various functional states of monocytes and macrophages are only now coming to be understood. Nevertheless, there is a growing awareness and appreciation of the potential metabolic and functional diversity which these cells can exhibit. Cohn has recently suggested a temporal sequence for the transition of monocytes from a resting, 'unactivated' state to an 'activated' state culminating in microbicidal or tumoricidal activity [10]. The early, nonspecific metabolic changes seen involve increased glucose oxidation, superoxide anion (O_2^-) production and hexose monophosphate (HMP) shunt activity. Those changes are associated with phagocytic activity and with a series of enzymatic changes in the cell and in its secretory capabilities. Subsequently, H_2O_2 production and microbicidal or tumoricidal function is expressed by what may be a lymphokine-mediated event.

The metabolic states of monocytes, particularly those states associated with the early phases of monocyte activation, have not been well studied in human cancer patients. One means of studying metabolic changes in monocytes which has seen increasing application in recent years is the measurement of monocyte chemiluminescence [16, 20, 25]. This phenomenon was originally described in phagocytically active polymorphonuclear leukocytes [5] and is thought to result from the relaxation of electronically excited molecules produced in association with oxidative metabolic events [4]. It can be correlated metabolically with oxidation of glucose via the HMP shunt [1, 11]. We have measured the chemiluminescence activity of resting and stimulated peripheral blood mononuclear cells obtained from normal subjects and from solid tumor patients with either advanced disease or at most minimum residual

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disease. The effect on chemiluminescence of administering Freund's complete adjuvant (FCA) alone or emulsified with extracted tumor-associated antigens (TAA) to lung cancer patients was also evaluated in this study.

Methods

1. Patient Population. The patient population consisted of 28 solid tumor patients, 14 classified as having advanced disease [solid tumor patients with stage IV disease and also glioblastoma multiforme (glioma) patients] and 14 as having at most minimum residual disease. Those patients with advanced disease were studied prior to initiation of any form of cytoreductive therapy. Five of the patients in that group had biopsy-proven gliomas and were studied prior to surgical exploration and diagnosis. Each of those patients was receiving dexamethasone at the time of immunological testing. Patients with minimum residual disease included stage II breast cancer patients (with axillary lymph node involvement by tumor) and stage I lung cancer patients both groups studied 10–30 days following definitive surgical resection of disease and prior to initiation of either adjuvant chemotherapy for breast cancer or adjuvant immunotherapy for lung cancer. Lung cancer patients entered for study of adjuvant immunotherapy were randomized to receive no further treatment, 3-monthly injections of FCA, or 3-monthly injections of FCA + TAA [28]. In those patients chemiluminescence was measured prior to vaccination and skin testing and 2 months following entry on study. The control population consisted of 16 healthy individuals, none of whom was receiving any medication.

2. Isolation of Peripheral Blood Mononuclear Cells (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 Mechanism (LSM, Bionetics, Kensington, MD) and centrifuged at 480 *g* for 20 min at room temperature. Platelets were removed by slow centrifugation in 0.14 *M* NH_4Cl. Isolated PBMC were washed twice in Hank's balanced salts solution, monocytes were enumerated by latex ingestion, and subsequently diluted in phosphate-buffered saline for further testing. Smears were made of isolated PBMC and stained with Wright's stain to determine percentages of lymphocytes, monocytes and other cell types, such as granulocytes, in the mononuclear cell preparation. Individual patients and patient samples were rejected from inclusion in this study when any sample contained 5% or more granulocyte contamination or platelet count greater than that seen in normal PBMC preparations. Those 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.

3. Monocyte Chemiluminescence Assay. The monocyte chemiluminescence assay was a modification of the procedure described by Allen [2]. Glass scintillation vials containing 1 nmol luminol were dark-adapted for 1 h. PBMC adjusted to contain 1×10^5 mononuclear phagocytes in barbital buffer were then added in a total volume of 2.0 ml and chemiluminescence was monitored at ambient temperature for 1–2 h in a Packard scintillation spectrometer in the out-of-coincidence mode. In some experiments, serum (25 µl) was also included in the reaction mixture. When stimulated chemiluminescence was measured, only 7×10^3 monocytes/vial were used. Background chemiluminescence was monitored as described above for 10 min, then 10 µg phorbol myristic acetate (PMA) was added, and light emission was monitored for 1–2 h.

4. Assays for Circulating Immune Complexes (IC). The Raji cell radioimmunoassay (RIA) for IC in serum was performed by the method of Theofilopoulos et al. [30]. RIA for C1q binding IC was performed by a modification of the method of Hay et al. [13], 3.5 mm polystyrene beads being used as the solid phase C1q absorbant.

Results

1. Evaluation of Chemiluminescence (CL) in Resting PBMC of Normal Subjects and Solid Tumor Cancer Patients

PBMC from normal subjects and from cancer patients were assessed for their baseline levels of CL. In these tests, the CL from PBMC adjusted in concentration to contain 1×10^5 monocytes was measured following dark adaptation of the cells for 1 h. Measurements were made following dark adaptation and at 20-min intervals thereafter for 80 min. The values for CL expressed represent the average of four determinations; the standard error of the mean rarely exceeded 10% (Table 1). When baseline CL levels for normal subjects were compared as a group with levels from patients with advanced disease, a significant difference between the two groups was found (mean ± standard deviation = 6,823 ± 3,175 cpm and 23,324 ± 9,976 cpm, respectively; $P < 0.001$, Table 1). On the other hand, patients with at most minimum residual disease as a group exhibited levels of baseline CL comparable to the control population (mean ± SD = 6,827 ± 2,073 cpm; not significant, Table 1). Patients were considered to exhibit levels of CL which were significantly different from normal when the CL value determined for an individual patient fell greater than 2 SD above the mean CL levels determined for the control population. By that criterion, 13/14 patients with advanced malignant disease exhibited CL levels that were significantly greater than normal (all except patient 6, Table 1), while all patients with minimum residual disease exhibited normal baseline CL values. When monocyte percentages determined for isolated PBMCs from normal subjects were compared as a group to those values in cancer patients, a significant increase in monocyte percentages was seen in patients with advanced disease (mean ± SD = 14.7 ± 3.6 for controls, vs 22.6 ± 8.8, $P < 0.001$), while no increase was seen in patients with at most minimum residual disease (mean ± SD = 15.2 ± 4.6, not significant [NS]). However, when monocyte percentage was correlated to baseline CL levels by linear regression

Table 1. Baseline chemiluminescence (CL) levels in PBMC from normal subjects, advanced cancer patients, and minimum residual disease cancer patients

	Normal subjects		Advanced cancer			Minimum residual disease		
	CL (cpm)	Monocytes (%)	Tumor	CL (cpm)	Monocytes (%)	Tumor	CL (cpm)	Monocytes (%)
	13,765	16	1. Lung	21,250	25	15. Breast	9,400	18
	4,824	11	2. Colon	20,500	45	16. Breast	5,575	16
	9,616	12	3. Glioma	18,875	19	17. Breast	9,044	24
	7,691	17	4. Glioma	20,714	12	18. Breast	5,953	12
	7,869	18	5. Colon	20,506	26	19. Lung	8,825	14
	4,808	14	6. Glioma	10,389	15	20. Lung	7,548	11
	4,942	8	7. Glioma	28,350	20	21. Lung	4,400	15
	12,992	16	8. Lung	20,110	20	22. Lung	7,903	13
	6,650	15	9. Breast	31,976	20	23. Lung	3,850	11
	3,983	13	10. Lung	51,028	24	24. Lung	5,600	16
	6,370	10	11. Melanoma	20,312	17	25. Lung	8,063	24
	3,644	18	12. Lung	16,933	37	26. Lung	5,410	12
	8,923	22	13. Glioma	31,500	16	27. Lung	4,131	18
	5,149	18	14. Melanoma	14,102	20	28. Lung	9,871	9
	4,930	12						
Mean ± SD	6,823 ± 3,175	14.7 ± 3.6		23,324 ± 9,927	22.6 ± 8.8		6,827 ± 2,073	15.2 ± 4.6

Table 2. Stimulation of monocyte chemiluminescence (CL) in PBMC from normal subjects and cancer patients by phorbol myristic acetate (PMA)

Normal subjects CL (cpm)	Advanced cancer	CL (cpm)	Minimum residual disease	CL (cpm)
70,846	1	172,000	13	188,692
26,114	2	35,575	14	122,000
32,067	3	93,725	15	72,735
18,645	4	14,725	16	475,034
22,280	5	215,001	17	71,138
26,500	6	137,335	18	19,785
26,914	7	176,388	19	75,765
68,000	8	81,430	20	36,430
27,110	9	127,951	21	42,364
46,938	10	225,063	22	82,000
31,780	11	127,992	23	62,077
22,306	12	29,616	24	49,000
			25	87,327
Mean ± SD 34,963 ± 17,602		119,730 ± 70,666		106,488 ± 118,651

analysis, no significant correlation was found ($r = 0.27$, -0.025, and -0.17 for normal controls, advanced cancer patients, and minimum residual disease patients, respectively).

2. Evaluation of CL in PMA-stimulated Monocytes from PBMC of Normal Subjects and Solid Tumor Cancer Patients

The capacity of monocytes from normal subjects and from cancer patients to be stimulated to produce CL was also evaluated in this study. In these tests, PBMC were adjusted in concentration to contain 7×10^3 monocytes, dark-adapted until stable baseline CL values were observed, and subsequently stimulated with 1×10^{-6} *M* PMA. CL measurements were taken at 20-min intervals following PMA addition generally for 1.5–2.0 h, which permitted peak CL levels to be achieved and subsequent subsiding of the response to be seen. When normal subjects were compared with cancer patients as a group, both patients with advanced disease and patients with minimum residual disease exhibited significantly greater PMA-stimulated peak CL levels than did normal subjects (mean ± SD = 34,963 ± 17,602 cpm, 119,730 ± 70,666

cpm, and 106,48 ± 118,651 cpm for normal subjects, advanced cancer patients, and minimum residual disease patients, respectively; $P < 0.001$ and 0.05, Table 2). As before, PBMC from patients were considered to produce significantly greater PMA-stimulated CL than PBMC from normal subjects when the patient value fell greater than two standard deviations above the mean stimulated CL level determined for the control population. By that criterion, 9/12 patients with advanced cancer and 8/13 patients with minimum residual disease exhibited significantly greater PMA-stimulated CL than normal subjects (Table 2). By linear regression analysis, there was no correlation between monocyte percentage and PMA-stimulated CL levels in either normal subjects or cancer patients ($r = 0.25$, -0.22, and -0.11 for controls, advanced cancer patients, and minimum residual disease patients respectively).

Table 3. Effects of in vivo administration of FCA alone or in emulsification with extracted lung tumor antigens on monocyte CL in PBMCs from lung cancer patients

Treatment group	Patient	Baseline chemiluminescence (CPM)[a]	
		Pretreatment	Posttreatment[b]
Control	1	7,917	5,328
	2	7,548	5,849
	3	12,219	5,946
FCA	4	7,903	17,734
	5	4,695	14,490
	6	10,356	16,140
	7	7,390	26,260
FCA + TAA	8	5,410	15,875
	9	6,569	19,420
	10	6,953	19,017

[a] Chemiluminescence from 1×10^5 monocytes in isolated PBMC
[b] Values obtained 2 months after entry on study

3. Assessment of the Influence of Vaccination with FCA or FCA + TAA on PBMC CL in Stage I Lung Cancer Patients

PBMC from stage I lung cancer patients who were entered on a program of adjuvant immunotherapy following surgical resection of disease were tested for CL activity prior to and following vaccination. Patients received either no further therapy, 3-monthly injections of FCA, or 3-monthly injections of FCA + TAA. All patients were skin tested with lung cancer antigens and with recall antigens (PPD, Candida, SK-SD) at the time of randomization for entry on study. Baseline CL levels were measured for patients prior to skin testing and 2 months following either skin testing alone or skin testing and first vaccination. Thus far, three control patients, four FCA patients, and three FCA + TAA patients have been studied in this manner (Table 3). Initially, cells from each patient exhibited baseline CL levels which were within normal limits. Two months later, the control patients who were skin-tested at the time of randomization again exhibited normal baseline CL levels. In contrast, the patients who were vaccinated with FCA and the patients who were vaccinated with FCA + TAA demonstrated significantly elevated baseline CL levels compared with normal at the post-skin testing, post-vaccination assessment point.

Table 4. Effect of serum surgically resected stage I lung cancer patients vaccinated with FCA + TAA on baseline CL levels of normal monocytes

Normal monocytes +	No. of donors	Immune complexes (mg Eq. aggregated IgG)[a]	Baseline chemiluminescence (Mean cpm ± SD[b])
Normal human serum	4	< 10	5,660 ± 1,172
Control patient serum (pre-skin test)	3	32 ± 14	5,505 ± 1,948
Control patient serum (pre-skin test)	3	29 ± 11	5,180 ± 756
FCA patient serum (pre-vaccination)	4	20 ± 8	7,220 ± 4,333
FCA patient serum (post-vaccination)	4	23 ± 10	40,520 ± 10,709
FCA + TAA patient serum (pre-vaccination)	3	43 ± 12	7,094 ± 1,690
FCA + TAA patient serum (post-vaccination)	3	41 ± 16	45,180 ± 9,750

[a] Values given determined by Clq test; Raji cell assay essentially negative in all cases
[b] Standard deviation

4. Assessment of the Influence of Serum from Lung Cancer Patients Receiving FCA or FCA + TAA on Baseline CL Levels of Normal Monocytes

Sera from lung cancer patients entered on studies of adjuvant immunotherapy were tested for their effect on baseline CL levels of normal PBMC. Sera from patients who were skin-tested alone or in conjunction with vaccination were added to PBMC from normal subjects containing 1×10^5 mononuclear phagocytes, and CL levels were measured as before (Table 4). CL levels of normal monocytes were comparable and within normal limits in the presence of 1) normal human sera from four different donors, 2) sera from control lung cancer patients collected both prior to and 2 months following skin testing, and 3) sera from vaccinated lung cancer patients collected prior to skin testing and vaccination. On the other hand, the addition of sera collected from patients 2 months following first vaccination to normal PBMC dramatically enhanced baseline CL levels from $5,660 \pm 1,172$ cpm in the presence of normal sera to $40,520 \pm 10,709$ and $45,180 \pm 9,750$ cpm in the presence of post-vaccination sera from FCA- and FCA + TAA-treated patients, respectively ($P < 0.01$). The factor(s) responsible in lung cancer patient sera for augmented CL of normal PBMC did not appear to be correlated with immune complex levels as measured in the C1q-binding assay since those levels were comparable in all patient groups at each assessment point (Table 4). Similarly, IC as detected by Raji RIA, were absent in both the pre- and the post-immunization sera.

Discussion

The results of this study demonstrated that patients with advanced malignant disease exhibit greater levels of CL in their resting peripheral blood mononuclear cells (PBMC) than are seen in PBMC from normal subjects and cancer patients with minimum residual disease. Greater stimulation of CL by PMA was seen with cells from cancer patients in states of both advanced or minimum residual malignant disease than with cells from normal subjects. Treatment of surgically resected stage I lung cancer patients with FCA alone or in combination with extracted TAA was found to increase PBMC CL levels by a mechanism which may have been mediated by a serum factor or factors. There was no correlation between CL and immune complex concentration with the serum studied. There was no apparent correlation between monocyte percentages in normal or patient PBMC and CL levels in this study.

The levels of CL seen in PBMC from normal subjects and cancer patients is presumed to reflect primarily the metabolic state of the mononuclear phagocytes contained within the test sample. We do not, however, discount the possibility that other cell types may contribute to the findings in this study. Particularly in the case of PBMC preparations from patients with disseminated cancer, the CL levels seen could reflect actions of lymphocytes, platelets, and/or granulocytes, apart from monocyte metabolism. We attempted to minimize that possibility by excluding from the study PBMC preparations which contained greater than normal levels of platelet or granulocyte contamination. The question of the impact of differing lymphocyte numbers in PBMC preparations on monocyte CL is a more complex issue. Our first approach to this problem was to attempt to isolate adherent cells prior to measurement of CL. We found that adherence of monocytes to glass surfaces led to substantial increases in CL of normal cells; thus, we rejected that approach. We then attempted to alter mononuclear cell numbers to determine the relationship between monocyte number and CL for any individual sample. We standardized the technique employed in the present study so that the monocyte numbers were linearly related to the CL seen in both normal subjects and cancer patients. Above 300,000 monocytes/vial that linear relationship did not hold. We also confirmed that under the conditions employed, glass nonadherent cells did not chemiluminescence. We considered the possibility that variable lymphocyte numbers in the assay could lead to significant alterations in detectable monocyte CL but would discount that possibility since 1) PBMC from normal subjects and/or cancer patients which contained identical monocyte and lymphocyte percentages exhibited substantial differences in CL activity (i.e., when comparing control no. 3 to control no. 15 or control no. 13 to patients 10 and 25); 2) varying the number of PBMC employed in the assay revealed a linear relationship between CL and monocyte numbers up to 300,000 monocytes/vial even though absolute lymphocyte numbers were increased in these experiments more than monocytes. Still, we do not totally exclude the possibility that cell types other than monocytes can contribute to the CL activity of PBMC preparations from cancer patients.

There is a substandial body of evidence which suggests that CL provides a reliable, quantifiable assay of oxidative metabolism in reticuloendothelial cells [1, 3, 4, 6], and with appropriate modifications, can also be used to measure particle uptake [12] or

serum opsonic activity [1, 2]. Macrophage activation has been assessed by means of CL [25], and since human monocytes also respond with light emission to a variety of stimuli [16, 20], it is reasonable to suggest that the enhanced CL from the PBMC of patients with advanced cancer reflects an in vivo stimulation, although CL alone cannot provide any direct information on the nature of the stimulus.

The increase of baseline CL in PBMC from patients with advanced malignant disease may be attributable to a variety of factors, including ongoing immunological reactions to tumor and/or stimulatory influences of tumor-derived products. In a portion of those patients, namely the patients with glioblastoma multiforme, the elevated CL levels seen may have reflected the influence of steroid treatment. On the other hand, we have observed normal CL levels in some treated glioma patients who were also receiving dexamethasone at the time of testing (D. P. Braun et al., unpublished data). The nature of the immune reactions contributing to the CL of patient cells cannot be defined by this study. Responses favorable or unfavorable to the host could be involved. Both specific and nonspecific immune reactions are likely to occur. Also, the possibility that tumor-produced effects contributed to the elevated baseline CL levels seen with PBMC from patients with advanced cancer is suggested by the fact that resting cells from patients with at most minimum residual disease did not demonstrate elevated baseline CL. On the other hand, PBMC from the majority of patients with either advanced disease or minimum residual disease appeared to have greater potential to become stimulated by PMA to produce CL. In patients with minimum residual disease, that might be at least partially due to residual inflammatory effects following surgery and indeed, all stage I lung cancer patients who received no further therapy were found to exhibit normal CL levels within 1–2 months following surgery. In patients with advanced disease, however, the increased capacity of patient cells to be stimulated by PMA to produce CL is most probably attributable to the influence of disease processes.

Higher baseline levels of CL in patients with advanced disease may indicate greater in vivo stimulation of monocytes and/or their precursors than that which affects cells from normal subjects or patients with minimum residual disease. Though this greater level of baseline CL was associated in advanced cancer patients with an increased percentage of monocytes in their PBMC, no significant correlation between monocyte percentage and CL level could be established. That lack of correlation held when normal subjects and patients with minimum residual disease were considered as well. On the other hand, the apparent increased potential of cells from the majority of cancer patients to be stimulated by PMA regardless of stage of disease suggests metabolic differences between PBMC from normal subjects and cancer patients. That observation has been made in several animal tumor systems and in human lymphoreticular malignancies by other methods [9, 18, 26].

Treatment of surgically resected lung cancer patients by FCA with or without tumor antigens was found to elevate CL levels in patient PBMC. That observation is consistent with the notion that agents which contain mycobacteria modulate the immune response partly through activation of monocytes [14, 19, 23]. It is of interest to note that increased monocyte percentages were not seen in patients following vaccination (data not shown). Therefore, elevated CL responses did not appear to coincide with increased production of monocytes in those individuals, but rather suggests a metabolic change in their cells. Those changes may have been produced by a serum factor or factors since serum from patients treated by vaccination markedly enhanced baseline CL levels of normal resting PBMC. Meltzer and Stevenson have reported that the increased phagocytic capacity of macrophages from tumor-bearing mice can be transferred to normal mice by serum [17]. Whether there is any relationship between the effect of vaccination on CL and the therapeutic effects attributed to those agents [28] requires further study.

IC may activate macrophages [22] and IC are often present in the sera of cancer patients [29]. We demonstrated that serum from patients with SLE which contained IC did produce increased CL in normal PBMC (Table 4). We measured IC levels in cancer patient sera with both the Clq and the Raji IC assay. We found no correlation between the presence of IC in vaccinated cancer patient sera and the ability of that sera to produce increased CL in normal monocytes. The levels of IC remained constant in pre-vaccination and post-vaccination sera but the post-vaccination sera produced increased CL, whereas pre-vaccination sera did not.

At present, the relationship of monocyte CL levels to discreet monocyte functional activities apart from phagocytosis is not known. We have investigated, in preliminary studies, the relationship of monocyte CL to monocyte suppressor function in cancer patients. In those studies, monocyte CL was not consistently associated with monocyte suppressor function. Moreover, monocyte CL levels were not significantly affected by indomethacin, a treatment which abolished monocyte suppressor function [7]. Further studies are now being conducted to elucidate

the relationship of monocyte CL to other monocyte functions in cancer patients.

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