Immunology of Tumor Infiltrating Lymphocytes

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Frequently peripheral blood lymphocytes (PBL) do not reflect the tumor host relationship and cell mediated immunity in the PBL does not often correlate with prognosis. The tumor infiltrating lymphocytes (TIL) interact most closely with the tumor cells and are likely to more accurately reflect tumor host interactions. These studies indicate that TIL from pulmonary tumors are similar to PBL so far as their cell surface markers are concerned. The percentage of T-cells, B-cells, helper cells, suppressor cells, and NK cells are similar in the two compartments. However, the TIL are markedly suppressed in their functional capacity as measured by their proliferative and cytotoxic activity. In addition, natural killer (NK) cell activity is markedly diminished in TIL as opposed to the PBL. In addition, the direct injection of BCG into these tumors reverses this phenomenon by significantly increasing T-cell and NK cell functional activity. Thus, the microenvironment of the tumor profoundly affects the immunologic relationship between the tumor and the host.

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THERE IS A large body of literature that has attempted to define and characterize the human tumor immune response.[5,14,15] These studies have demonstrated tumor associated antigens and cellular and humoral immune responses in the peripheral blood directed towards these antigens. In some instances, brisk immune responses to tumor antigens can be detected in patients with malignancy and in other instances these responses cannot be demonstrated. The inability to detect immune responses in these patients has been explained by a general depression in cell mediated immunity in patients with cancer.[4] However, on balance, these studies of peripheral blood lymphocytes and serum have given very inconsistent results. Furthermore the reactivity of peripheral blood lymphocytes and antibodies does not correlate with survival. This paradox has been particularly frustrating to immunologists and clinical immunotherapists.[1,2,7]

The appreciation that the lymphocyte population is quite heterogenous consisting of many different clones of lymphocytes containing a variety of cell surface markers led to the notion that these subpopulations may not be uniformly distributed throughout the body. Thus the concept of compartmentilization of the immune system evolved. Studies in animal tumor models and a few observations in man suggest that the inflammatory cells infiltrating the tumor more accurately reflect the tumor host relationship than do the peripheral blood lymphocytes.[18,6,10] Therefore the detection of cytotoxic effector cells in sites distant from the tumor does not necessarily indicate that the same reactive cells are coming into direct contact with the tumor. Studies in animal tumor models have indicated that progressively growing tumors contain weak or nonreactive tumor lymphocytes but that regressing tumors have highly reactive lymphocytes.[1] These immunologic reactions within the tumor are quite different from the lymphocyte reactivity in the regional lymph nodes and the peripheral blood. In human studies most tumor infiltrating lymphocytes (TIL) are immunologically nonreactive despite the presence of reactivity in the peripheral blood lymphocytes (PBL) and the regional lymph node lymphocytes.[10,13]

We have attempted to better define the nature of these tumor infiltrating lymphocytes (TIL) in pulmonary tumors. Our studies have indicated that lymphocytes infiltrating human pulmonary tumors are quite immunosuppressed and are essentially paralyzed in terms of their functional activity. While this situation pertains in the microenvironment of the tumor, in the same patient the peripheral blood lymphocytes are quite reactive. We have attempted to define the mechanism of this immunosuppression within the tumor. In addition, preliminary studies have indicated that the direct injection of bacillus calument Guerin (BCG) is capable of reversing this immune paralysis.

Materials and Methods

Specimens

Surgical resected specimens of patients with primary and metastatic cancer were studied. Primary lung cancers were adenocarcinoma and squamous carcinoma. Metastatic tumors included soft tissue and bone sarcomas,

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hypernephroma, colon cancer, as well as metastatic melanoma. Peripheral blood was also obtained from each of these patients in order to evaluate the peripheral blood lymphocytes as well as the tumor infiltrating lymphocytes. In those patients undergoing intralesional BCG injection, Glaxo BCG 10^5 organisms in 0.5 ml of sterile water were injected percutaneously under fluoroscopic control 14 days before operation as previously described.[8]

Preparation of TIL

Tumor infiltration lymphocytes (TIL) were purified as described previously.[12] Extraneous normal and necrotic tissues were removed from the fresh tumor. The specimens were then minced and mixed with RPMI-1640 media supplemented with 10% fetal calf serum. After washing, the cells were placed on a Ficoll-Hypaque gradient for discontinuous density gradient separation. Dead cells, erythrocytes, granulocytes, and most of the tumor cells pelleted to the bottom and the cells at the interface consisting of lymphocytes, monocytes, and macrophages, and some tumor cells were collected. The cells at the gradient interface were washed with RPMI-1640. The macrophages were removed by adding carbonyl iron, incubating at 37 C for 30 minutes and then centrifugation for 10 minutes at 2000 g. The supernate that was macrophage-free was collected and density gradient cell separation was performed on a Wescor Model 6000 CelSep. The details of this separation technique have been previously described.[12] After the appropriate fraction was collected, the fraction was centrifuged at 2000 g for 10 minutes and resuspended in 10% FCS. Cell number and viability were determined by trypan blue dye exclusion.

Peripheral blood lymphocytes (PBL) were obtained from Ficoll-Hypaque gradients as previously described.[13]

MCL-CML Assay

An allogeneic lymphoblastoid cell line (LCL) was used as the stimulator cell in the MLC assay. 10^5 responding lymphocytes were co-cultivated with 2.5 $\times$ 10^4 irradiated LCL. Each assay was performed in quadruplicate. Control assays include responder PBL from a healthy donor. After 5 days of incubation, 0.5 μCi of titrated thymidine was added for an additional 18 hours. Following harvesting, the cells were placed in a liquid scintillation counter for counting. The LCL also served as the target cells in the CML assay. Six days after the original initiation of cultures, the target cells were labeled with 250 μCi of 51_{Cr} and incubated for 1 hour at 37 C. The cells were then resuspended at two concentrations (5 $\times$ 10^5 ml and 1.25 $\times$ 10^5 ml) in medium containing 20% pooled human AB serum. The two concentrations of targets were separately added to wells of microtiter plates. Final effector-to-target cell ratio were 4:1 and 32:1. After incubation for 5 hours the plates were centrifuged and the supernatant collected and counted on a gamma counter. Cytotoxic activity was calculated as follows: Per cent cytotoxicity = (release in test samples − spontaneous release)/(total release − spontaneous release) $\times$ 100.

NK Cell Activity

NK cell activity was evaluated by a chromium release assay and also by the single cell assay. In the chromium release assay K 562 target cells were tested with a 4-hour chromium release microassay. All experiments used 50:1 responder-to-target ratios. The results were expressed as a percentage cytotoxicity by the same formula as used in the CML assays.

The single cell lysis and binding assay for NK cell was performed as described by Grimm and Bonavida with modifications by Targan et al.[3,16] Briefly, 5 $\times$ 10^5 K562 target cells were mixed with 5 $\times$ 10^5 lymphocytes and incubated for 10 minutes at 37 C. After centrifugation for 4 minutes at 800 rpm, an equal volume (100 microliters) of warm (37 C) 2% agarose solution was added. The resulting mixture was then spread on slides previously coated with agar. The slides were then incubated in RPMI-1640 for varying periods at 37 C, placed in 1% trypan blue for 10 minutes, rinsed for 10 minutes, and fixed in 0.1% formalin solution. Slides were scored for percentage of lymphocytes binding to K 562 targets (200 lymphocytes scored) and percentage of target binding cells able to mediate lysis (100 binders scored).

Determination of Intrinsic Cytotoxic Capability

The inherent lytic capacity was determined by utilizing an applied Michaelis-Mendin enzyme model as described by Ullberg et al.[17] Briefly a 51_{Cr} release assay was utilized using varying concentrations of K 562 target cells against a fixed number of effector cells (10^5). Using a linear regression analysis, a theoretical V_{max} was determined as the K 562 target concentration approached saturation kinetics, thus reflecting the inherent lytic capability for a fixed number of lymphocytes.

Determination of Lymphocyte Phenotypes

The proportion of T-cells and complement receptor cells (B-cells) were identified by E-rosette and EAC-rosette formation, respectively. The technique was as previously described.

Lymphocyte phenotypes were also determined by a universal rosetting reagent and murine monoclonal antibodies as previously described by Kaivodin and Golub.[8]

TABLE 1. *Surface Markers by Rosettes (per cent)*

	E	EAC
TIL	27.3 ± 2.7	12.0 ± 2.6
(n)	(30)	(30)
PBL	32.5 ± 3.3	8.6 ± 2.8
(n)	(23)	(23)

Per cent E and EAC rosetting lymphocytes in TIL and PBL.

Briefly, a rosetting reagent was constructed using oxerythrocytes that were treated with rabbit anti-ox RBC IgG antibody. After Staphyloccus protein A was bound to the antibody-coated RBC, rabbit anti-mouse IgG antibody was added. Lymphocytes were incubated with various mouse antibodies with specificity directed against the lymphocyte markers of interest and washed. The universal rosetting reagent was then added. After incubating for 1 hour at 4 C, samples were resuspended and placed on glass slides for scoring. At least 200 lymphocytes were scored to determine the per cent rosetting.

Results

The lymphocyte phenotypes or cell surface markers did not differ significantly in the TIL population and the PBL population. Table 1 indicates that the TIL contained 27% E-rosetting lymphocytes, whereas the PBL contained 32% E-rosetting lymphocytes. The EAC receptors (B-cells) represent 12% of the lymphocyte population in TIL and 8.6% in the PBL. Therefore the T-cell population and the B-cell population did not differ significantly between the TIL and PBL. In addition, the cell surface markers as determined by the various monoclonal antibodies illustrated in Table 2 indicates that the monoclonal antibodies directed against the Fc receptor and NK cells are similar in both the PBL and TIL. Similarly, monoclonal antibodies to NK cells, T-cells, helper cells, and suppressor cells demonstrated an equal distribution of these lymphocyte phenotypes in both the PBL and TIL.

The functional reactivity of the TIL and PBL was evaluated in the MCL-CML reaction. The MLC reaction tests the proliferative capacity of the lymphocytes and the CML reaction assesses the ability of the T-cells to develop into cytotoxic effector cells. As Table 3 indicates, the TIL have a marked decrease in their proliferative capacity (MCL) as well as their cytotoxic capacity (CML) when compared to the PBL. When expressed as the per cent of control reactivity, the TIL had only a 6.5% reactivity in MLC and 13.8% in CML. This was in contrast to a 77.9% reactivity of the PBL in MLC and 88.8% in CML. Therefore, TIL were shown to have a marked suppression of their functional reactivity in MCL-CML.

Since NK cells are felt to be important in tumor rejection, the NK activity of the PBL and TIL were evaluated. Two different assays were used to evaluate NK cell activity. In both assays the standard K 562 target cells were used. However, one assay employed chromium release as a sign of cell death and the second assay utilized vital dye staining in order to assess visually the ability of the NK cells to bind to the target cells as well as to destroy the target cell. As Table 4 indicates, the NK activity as measured by the chromium release assay was markedly decreased in the TIL compared to PBL. The PBL in these patients had a normal level of NK cell activity whereas the TIL had a marked suppression in NK cell activity ($p = 0.001$). In Table 4 the NK cell activity is assessed by the so-called single cell assay in which cell viability is determined by the ability of the viable cell to exclude the vital dye. Thus, in this assay the number of lymphocytes that are bound to a tumor cell can be evaluated by direct visualization and the number of tumor cells actually killed can also be evaluated. Surprisingly, the NK activity in the TIL and the PBL did not differ in this assay (Table 5). Fourteen per cent of the TIL exhibited binding and 14% of the PBL exhibited binding. In addition, 11% of the TIL as well as the PBL were capable of killing in this assay. Thus, on the one hand total killing by chromium release was suppressed in the TIL but binding and killing in the short term single cell assay was similar in the PBL and TIL.

In order to determine the inherent K 562 lytic capacity of the lymphocytes, a Michaelis-Mentin enzyme model as described by Ullberg et al. was applied. A fixed number of effector cells (1×10^5) were titrated against

TABLE 2. *Percentage of Cells Bearing Lymphocyte Markers as Determined by Monoclonal Antibodies*

	B73.1 (F_c and NK)	HNK-1 (NK Cell)	OKT3 (Pan T Cell)	Leu 3 (Helper)	Leu 2 (Suppressor)	Leu 3 / Leu2
PBL	13.5 ± 2.0	14 ± 2.4	51.7 ± 2.7	37.8 ± 3.6	16.6 ± 2.7	1.82 ± 0.4
(n)	(11)	(10)	(11)	(11)	(10)	(10)
TIL	10.0 ± 7.7	19.6 ± 3.6	44.7 ± 6.7	39.6 ± 10	36.3 ± 4.4	1.34 ± 0.4
(n)	(12)	(9)	(6)	(5)	(6)	(5)

Per cent of cells bearing lymphocyte markers in TIL and PBL. There are no significant differences.

TABLE 3. *Functional Activity in MLC-CML (Per cent Control)*

	MCL	CML
TIL	6.5 ± 2	13.8 ± 5.9
(n)	(24)	(24)
PBL	77.9 ± 15.3	88.8 ± 11
(n)	(20)	(20)
	p = <0.005	p = <0.005

Counts per minute (CPM) and per cent of normal control lymphocyte activity in the MLC-CML assay of TIL and PBL. Both MLC and CML are significantly lower in the TIL ($p < 0.005$).

TABLE 5. *Single Cell Analysis of TIL and PBL NK Cell Activity*

	TIL	PBL
Per cent binding*	14.2 ± 5.2	14.3 ± 4.6
Per cent killing†	11.2 ± 2.7	11.2 ± 3.5
(N=)	(18)	(15)

Per cent binding and killing by NK cells from TIL and PBL. No significant difference.

* Per cent lymphocytes binding K 562 targets, 200 lymphocytes counted.

† Per cent target binding cells mediating target lysis, 100 lymphocytes scored.

various concentration of K 562 cells. In this assay the maximum lytic capacity (V_{max}) of a fixed number of lymphocytes could be determined. Table 6 indicates the V_{max} for the TIL and PBL in this assay. These data show a significant decrease in the V_{max} or the inherent lytic capability of the TIL. Thus one could conclude that a single NK cell derived from the PBL is capable of killing far more target cells than a single NK cell derived from the tumor. Therefore, the single cell assay indicates that the number of NK cells actually present within the tumor and in the PBL are similar. However, the chromium release assay and the determination of the V_{max} indicates that the TIL NK cells have a defect in their lytic capability.

Table 7 indicates that following BCG injection, the proliferative capacity of the TIL is significantly increased compared to TIL of noninjected tumors ($p = 0.05$). Table 7 also indicates that the cytotoxicity of the TIL is enhanced by BCG injection compared to non-BCG injected TIL, but this difference does not quite reach statistical significance. The most dramatic effect of intralesional BCG injection is represented in Table 8. Four patients underwent direct injection of their pulmonary tumor prior to surgery. Following surgical removal of the tumor, NK cell activity was evaluated by the chromium release assay. Three of these four patients underwent resection of pulmonary metastatic lesions. Therefore, the TIL from the BCG injected tumor and the TIL from a non-BCG injected tumor from the same patient could be evaluated. Three unique compartments could be evaluated from the same patient: a tumor injected with BCG, a tumor in the same lung uninjected with BCG, and the PBL from the same patient. These studies indicated that BCG injection causes a striking increase in the NK cell lytic capacity in the BCG injected tumor whereas the non-BCG injected tumor from the same patient continues to have a depressed NK cell lytic capacity.

Discussion

These studies demonstrate marked differences between the peripheral blood lymphocytes and the tumor infiltrating lymphocytes and explain to a large extent why the tumor immune responses in the peripheral blood do not reflect prognosis. These studies have indicated that similar populations of lymphocytes infiltrate the tumor and the peripheral blood. The percentage of T-cells, B-cells, Fc receptor cells, NK cells, suppressor cells, and helper cells are similar in the two compartments. However, there is a striking difference in the functional capacity of these lymphocytes. Lymphocyte infiltrating tumors have a marked demunition in their proliferative and cytotoxic capacity. The single cell assay indicates that the percentage of NK cells in the TIL was not dissimilar to those in the PBL. On the other hand, the chromium release assay indicated that the NK cells that were present in the TIL had a marked deficiency in their ability to mediate lysis of tumor targets. Finally, preliminary studies evaluating the effect of the direct injection of BCG into human pulmonary tumors have

TABLE 4. *NK Activity ^{51}Cr Release*

	TIL	PBL
Per cent Cytotoxicity	6.6 ± 1.2	26.3 ± 3.0
(n)	(33)	(13)
	p = <0.0001	

Per cent of normal control NK activity in TIL and PBL by 51_{Cr} release ($p < 0.001$).

TABLE 6. *Comparison of NK Cytotoxicity and V_{max} of PBL and Autoclonous TIL in ^{51}Cr Release Assay**

	TIL	PBL	
Per cent cytotoxicity	4.9 ± 4.0	26.8 ± 15.9	$p < 0.001$
V_{max}	506 ± 468	6428 ± 6908	$p < 0.02$

Decrease cytotoxicity and V_{max} in TIL compared to PBL.

* Effector/Target 50:1.

TABLE 7. *Effect of BCG Injection on MLC-CML (Per cent Control)*

	MCL	CML
	(%)	(%)
BCG/TIL n = 11	23.2 7	22.1 13.1
TIL n = 24	6.5 2.01	13.8 5.9

BCG injection augments MLC and CML in TIL.

indicated that BCG injection is capable of enhancing significantly the MLC-CML response as well as significantly augmenting NK cell tumor lysis.

It is clear that the microenvironment of the tumor is inhibitory for a number of lymphocyte functions. Depression of various aspects of cellular immune activity among TIL has been reported by several groups.[1,10,13] Some investigators have found that TIL have suppressor cell activity that may account for their poor performance.[18] However, we have not been able to demonstrate suppressor cell activity in our studies. Others have reported that tumor cells *in vitro* are capable of secreting prostoglandins that may also lead to the inhibition of lymphocyte-mediated activity. We have also considered the possibility that residual tumor cell contamination in the TIL preparations may inhibit these responses. However, complete purification of the TIL preparation to eliminate contaminating tumor cells fails to restore the functional activity of the TIL.

There are several possible mechanisms for the low NK cell activity of TIL. Our studies have indicated that indeed NK cells are present in these tumors. In addition, our studies have indicated that the NK cells present in TIL are capable of binding to the target cells. Thus, the actual numbers of NK cells cannot account for the observed depression of the cytotoxic activity. In addition, this defect in NK activity was not due to inadequate recognition of target cells since binding was demonstrated. We have the observation that NK activity of TIL is low when measured both by the standard chromium release assay and when the total lytic capacity is estimated (V_{max}). However, the lytic activity appears to be normal in the single cell analysis. One explanation might be that NK cell infiltrating tumors have an impaired recycling capacity. These cells are able to mediate lysis of a single target but were unable to find or kill subsequent targets. Therefore, they would appear normal in the single cell assay but would have low activity in the chromium release assay. Impaired recycling capacity remains the most likely explanation for low NK activity in the TIL.

TABLE 8. *Effect of BCG Injection on NK Activity (^{51}Cr Release)*

	Per cent ^{51}Cr Release
TIL-BCG (n = 4)	24.8 ± 8.3
TIL (n = 3)	5.7 ± 4.7
PBL (n = 4)	36.5 ± 7.9

BCG injection augments NK activity in TIL.

The microenvironment of the tumor suppresses the overall cytotoxic efficiency of the NK cells as well as the proliferative capacity and cytotoxic capacity of the T-lymphocytes. The mechanism of this suppression remains unknown. It could be mediated by soluble substances within the tumor, suppressor cells, or both.

Regardless of the mechanism of suppression, it is clear that the direct injection of BCG into these tumors is capable of reversing this suppression and augmenting the functional capacity of the TIL. Our studies indicate that BCG injection increases the T-lymphocyte portion of the TIL and clearly augments the proliferative capacity as well as the cytotoxic capacity of T-cells as measured by the MLC and CML assay. The NK cell functional augmentation by intralesional BCG injection was particularly striking. NK cell function has been shown to increase following BCG administration in mice and humans. Our studies showed the BCG induced augmentation of NK activity can be manifested at the tumor site. Therefore, the local immunosuppressive environment of the tumor can be overcome, at least in part, by the direct injection of agents such as BCG. It is interesting that this augmentation appears to be confined to the BCG injected tumor since uninjected tumors in the same lung have very low NK activity. This observation indicates that systemically administered BCG would not have a similar effect as direct intralesional injection.

This immunosuppressive microenvironment may well function as a protective mechanism for the tumor. Peripheral blood lymphocytes finding their way into the tumor become paralyzed in this environment. Therefore, analyses of the peripheral blood lymphocyte *in vitro* do not reflect the *in situ* situation. The nature of these immunosuppressive factors remains unknown but it is encouraging that immunostimulators such as BCG are capable of reversing the suppression. Again, the mechanism of action of BCG in this situation is unknown. Other agents may be equally as effective, either given locally or systemically.

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