LOCAL XENOGENEIC GRAFT-*VS*-HOST REACTION: A PRACTICAL ASSESSMENT OF T CELL FUNCTION AMONG CANCER PATIENTS

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The local xenogeneic graft-*vs*-host reaction (XGVHR) was used as a practical bioassay to assess T lymphocyte function and immunocompetence among cancer patients. Positive XGVHR was found in 99.5% of normal donors, 70% of cancer patients with early stage disease, and 30% of cancer patients with metastatic disease (p < 0.001). A minimum of 4.5×10^6 immunocompetent T lymphocytes are necessary in order to elicit a positive XGVHR. Negative reactions among cancer patients are characterized by the lack of edema fluid accumulation and the appearance of the host basophils at the test site. This suggests that insufficient amounts of lymphokines are being released by the incompetent T lymphocytes, whereas the host is capable of mounting a rejection reaction as evidenced by the appearance of the basophils. Preliminary evidence suggests that the immunologic defect detected by the XGVHR cannot be corrected by monocyte depletion. The identification of putative suppressor T cell subsets may bear immunotherapeutic implications in the future.

The continuing quest for a single practical test that could faithfully assess the state of immunocompetence in cancer patients undergoing active treatment reflects the relative insensitivity, cumbersome methodology, or difficulty in interpretation and analysis of the currently used immunologic assays for the evaluation of the immune status. The most widely used skin tests for delayed hypersensitivity to primary and to recall antigens, and the *in vitro* blastogenic responses to mitogens have been rather disappointing mainly because there is certainly no clear-cut distinction between cancer patients and normal donors that could be consistently demonstrated on an individual basis.

The judicious management of the cancer patient will ultimately require a reliable and practical test, perhaps analogous to serum-creatinine determination for evaluation of kidney function, in order to evaluate general immunocompetence. We

have recently repeated and further developed the work by Shohat *et al.* (1–4) using the local xenogeneic graft-*vs*-host reaction (XGVHR)[2] in a fresh attempt to evaluate immunocompetence. We found that this is a highly reproducible, simple, and inexpensive immunologic bioassay testing several components of T cell function with a remarkable distinction between cancer patients and normal donors.

MATERIALS AND METHODS

Peripheral blood mononuclear cell preparations. Peripheral blood mononuclear cells were separated as previously described (5) from 212 healthy normal and blood bank donors and from 207 previously untreated patients with carcinoma of the large bowel, lung, and breast; melanoma, and multiple myeloma. The mononuclear cells were counted and resuspended in RPMI medium at a concentration of 10^8 cells/ml.

Peripheral blood monocyte preparations. The Ficoll-Hypaque-separated mononuclear cells were allowed to adhere to 150 x 15 mm plastic Petri dishes 1 hr at 37°C as previously described (6). The nonadherent cells were separated by gently washing six times with warm (37°C) RPMI medium supplemented with 10% fetal calf serum. The adherent cells (>98% monocytes identified by the esterase stain according to Yam *et al.* (7) and <2% lymphocytes) were then removed mechanically with a rubber policeman and were resuspended in RPMI medium at a concentration of 10^7 monocytes/0.1 ml.

T lymphocyte separation. The nonadherent lymphoid cells were mixed and incubated with sheep erythrocytes (SRBC) for E-rosette formation followed by differential sedimentation on Ficoll-Hypaque gradient as previously described (8). The supernatant containing chiefly B + "null" cells (<5% T cells) was removed and the rosetting SRBC at the pellet were lysed with ZAP-Isoton II (6 drops/20 ml) (Coulter Diagnostics, Hialeah, Fla.). The cell suspension containing >97% T lymphocytes (E-rosettes), 1% B lymphocytes (EAC rosettes), and 1% monocytes (esterase stain) was washed and resuspended in RPMI medium.

B lymphocyte preparation. The supernatant containing the mixture of B lymphocytes and "null" cells was mixed and incubated with SRBC coated with antibody and complement for EAC-rosette formation as previously described (8). The rosetting lymphocytes were separated by differential sedimentation on Ficoll-Hypaque gradient. the SRBC were lysed as described above, and the cell suspension containing 83 to 85% B lymphocytes, 15 to 17% monocytes, and <0.5% T lymphocytes was washed and resuspended in RPMI medium.

Preparation of "null" cells. The supernatant-containing cells remaining after E- and EAC-rosetting procedures, were considered "null" cells. These cell suspensions also contained <1% T

[2] Abbreviations used in this paper: XGVHR, xenogeneic graft-*vs*-host reaction.

lymphocytes, <1% B lymphocytes, and 2 to 3% monocytes.

Local GVH reaction procedure. Inbred Lewis male rats (Charles River Laboratories, Wilmington, Mass.) of approximately 150 g each were used. The animals were partially immunosuppressed by injecting 100 mg/kg of cyclophosphamide into the tail vein 24 hr before GVH testing. For the standard local GVH reaction, 2×10^7 human Ficoll-Hypaque-separated mononuclear cells in 0.1 ml medium were injected intradermally per site into the closely shaven abdominal skin of the rats. Smaller numbers of cells (5×10^6, 10^7) were injected when enriched subsets of mononuclear cells were used. Mononuclear cells inactivated by mitomycin-C (9) were used as negative controls. Six injection sites per rat were routinely used.

Although the GVH reaction could already be palpated 24 hr after cell injection, it reached its maximum at 48 hr when the rats were sacrificed, their abdominal skins were inverted inside out, and the local GVH reaction was directly visualized through the peritoneal lining. The reaction sites were carefully cut out and their volume (mm³) was calculated by the formula: $V = \frac{4\pi}{3} \frac{A}{2} \times \frac{B}{2} \times \frac{C}{2}$ where A, B, and C are the three right-angle diameters (in millimeters) of the ellipsoid nodule. A GVH test site of <50 mm³ is defined as a negative reaction, since 99.5% of the normal donor's cells produced GVH reactions >50 mm³ (Table I). A lesion of 50 to 250 mm³ is defined as 1+, 250 to 500 mm³ as 2+, and >500 mm³ is defined as 3+. In some studies we measured the radioisotope ¹²⁵I-uptake by the local GVH reaction after injection of ¹²⁵I-labeled bovine serum albumin (BSA, Sigma, St. Louis, Mo.), labeled with ¹²⁵I (New England Nuclear Corp., Boston, Mass.) as previously described (10). Next, 5.8 µCi/0.2 ml isotope-labeled BSA (specific activity 5.8 mCi/mg BSA) were injected into the tail vein 1 hr before sacrificing the animals. The GVH nodules were carefully cut out as described above, and the radioactivity incorporated in them was measured by a gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.) and expressed in counts per minutes (cpm). Histologic slides were routinely prepared from local GVH reactions. At least 10 microscopic fields in each slide were studied by light microscopy, with some of the specimens being coded. Results were analyzed statistically by the χ^2-test for comparisons of cancer patients *vs* normal donors and cancer patients *vs* cancer patients (Table I) and by the Student *t*-test for comparisons between normal donors' subpopulations (Table II).

RESULTS

Macroscopic and microscopic features of local graft-vs-host reaction. The macroscopic appearance of a positive local GVH reaction using normal donors' Ficoll-Hypaque-separated mononuclear cells is characterized by a local swelling with abundant edema fluid. In contrast to the striking appearance of the positive reactions, mitomycin C-treated mononuclear cells do not produce any visible reaction. Microscopically, the injected site showing a positive GVH reaction is characterized by a massive infiltrate of mononuclear cells around small blood vessels with an occasional mitotic figure.

In contrast to the mononuclear cells characterizing a positive GVH reaction, the negative controls using mitomycin C-treated cells to induce local GVH reactions are characterized by a distinctly different cellular infiltrate consisting mostly of polymorphonuclear cells and cellular debris.

Local GVH reaction: normal donors vs cancer patients. Local GVH reaction was tested among 212 normal donors and among 207 previously untreated cancer patients (Table I). All

TABLE I
Local GVH reaction: normal donors vs cancer patients

Category	No. Studied	GVHR Volume mm³, Mean ± S.D.	% Positive (>50 mm³)
Normal donors	212	142 ± 76	99.5
Cancer patients	207	52 ± 81	45
Early stage (local & regional) disease—usually NED	77	91 ± 74	70
Colorectal cancer, Dukes' C	50	98 ± 79	74
Melanoma, stage IIIB	21	75 ± 85	52
Breast cancer, stage II	4	94 ± 27	100
Multiple myeloma	2	72 ± 15	100
Far advanced or disseminated disease	130	31 ± 41	30
Lung cancer	90	21 ± 56	25.5
Colorectal cancer	12	31 ± 46	25
Breast cancer	10	61 ± 23	50
Multiple myeloma	13	59 ± 37	46
Melanoma	5	48 ± 50	40

TABLE II
Local GVH reaction with T-lymphocytes and combinations (summary of experiments in six normal donors)

Autologous Inoculum Admixed	GVHR Volume (mm³, mean ± S.D.) T Lymphocyte Inoculum (E-Rosettes)	
	5 × 10⁶	10⁷
None	76 ± 20	252 ± 29[a]
10⁷ Mononuclear cells (F-H)	251 ± 21	866 ± 17
10⁴ Monocytes ⎤	3 ± 2	163 ± 13[b]
10⁵ Monocytes ⎟ glass adherence	10 ± 5	172 ± 19[b]
5 × 10⁶ Monocytes ⎟	11 ± 14	195 ± 14[b]
10⁷ Monocytes ⎦	7 ± 10	146 ± 31[b]
5 × 10⁶ B lymphocytes ⎤ EAC rosettes	12 ± 4	267 ± 27
10⁷ B lymphocytes ⎦	7 ± 3	270 ± 33
5 × 10⁶ Null lympho- cytes ⎤ T, B, and monocytes	16 ± 18	257 ± 28
10⁷ Null lymphocytes ⎦ —depleted	4 ± 11	251 ± 25

[a] *vs.*
[b] $p < 0.005$.

but one of the normal donors (99.5%) showed a positive (>50 mm³) local GVH reaction, whereas only 93 of 207 (45%) cancer patients showed a positive reaction ($p < 0.001$).

Among the cancer patients, the incidence of positive local GVH reaction was 70% (54 of 77) for patients with early stage of local or regional malignant disease, as opposed to the incidence of 30% (39 out of 130 patients) of positive local GVH reaction among those with far advanced disseminated disease ($p < 0.001$).

Although the histologic appearance of the macroscopically positive local GVH reaction is the same for both normal donors and cancer patients, such is not the case when macroscopically negative GVH reactions are considered. While the macroscopically negative GVH reaction observed with mitomycin-C-treated normal mononuclear cells was characterized by the appearance of polymorphonuclear cells and cellular debris, the

microscopic appearance of a macroscopically negative reaction from a cancer patient is characterized by the mononuclear cell infiltration around the small blood vessels similar to the local GVH reaction with two apparent exceptions: 1) the total lack of edema fluid characterizing a positive GVH reaction macroscopically; 2) the presence of basophilic leukocytes in close proximity to small blood vessels.

Preliminary attempts to restore the GVH reaction from negative to positive by monocyte depletion before injection of grafted cells were unsuccessful. Thus, the mean GVH reaction among 10 patients with metastatic carcinoma of the lung, liver, and colon when using unseparated mononuclear cells was 8.6 ± 7.9 mm³, as compared to a mean of 9.2 ± 3.3 mm³ after monocyte depletion by glass adherence.

Local GVH reactions with subsets of mononuclear cells and combinations. To determine the subsets of peripheral blood mononuclear cells responsible for the normal local GVH reaction, enriched subpopulations of monocytes, T lymphocytes, B lymphocytes, and "null" lymphocytes from six normal donors were used separately and in multiple combinations (Table II). Positive GVH reactions were observed only with T lymphocytes. None of the non-T subsets injected either separately (in the same doses as for T lymphocytes) or in combination with other non-T subsets failed to show any visible local GVH reaction. Admixture of T cells with unseparated mononuclear cells increased the GVH reaction, probably by virtue of the increased numbers of T cells. Similarly, 10^7 mononuclear cells (containing approximately 50 to 60% T cells) produced GVH reactions that were consistently and significantly smaller than those observed with 10^7 T cells. Furthermore, it appears that some non-T subsets have an inhibitory effect on T lymphocyte-induced local GVH. The inhibitory effect is particularly pronounced for monocytes. Thus, monocytes in a wide range of doses (10^4 to 10^7) could partially but significantly ($p < 0.005$) inhibit the GVH induced by both the lower (5×10^6) and the higher dose (10^7) of T lymphocytes, whereas B and "null" lymphocytes were only inhibitory against the lower (5×10^6) dose of T lymphocytes.
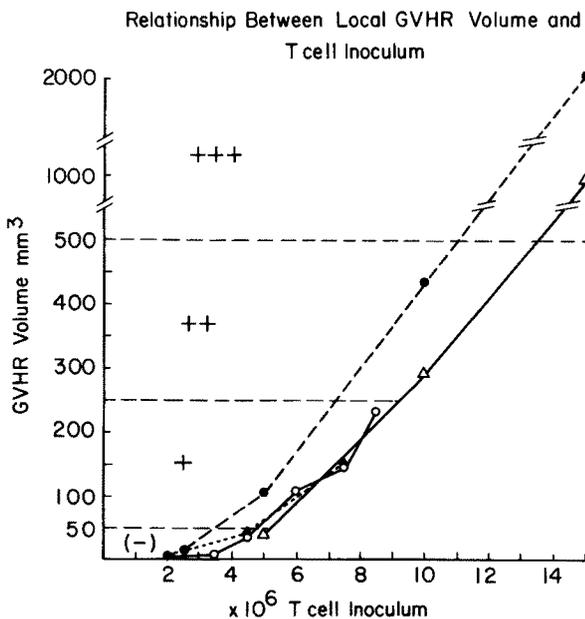

Figure 1. The relationship between T cell inoculum and volume of the local GVH reaction in four normal donors. A positive reaction (>50 mm³) requires a minimum of 4.5×10^6 immunocompetent T cells.
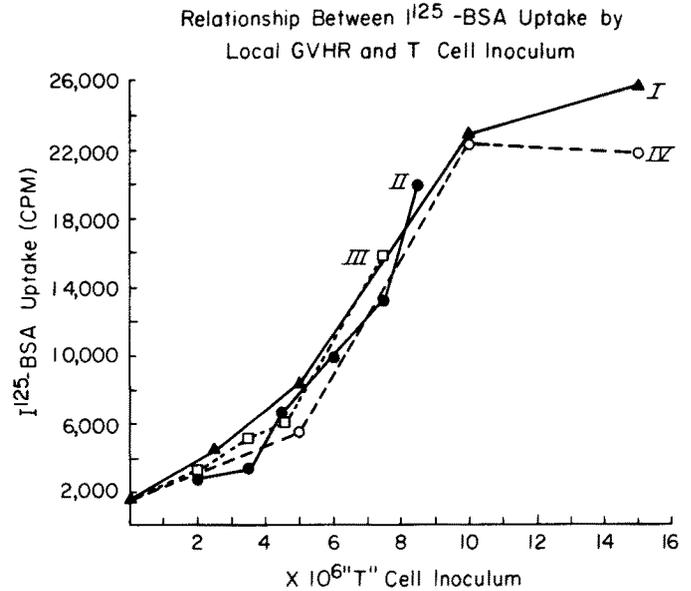

Figure 2. The relationship between T cell inoculum and I^{125} uptake by the local GVH reaction in four normal donors. Please note the linear relationship up to an inoculum of 10^7 T cells.

The intensity of T lymphocyte-induced local GVH reaction clearly appears to be dose related (Fig. 1). Inocula of $\geq 4.5 \times 10^6$ T lymphocytes regularly produced positive local GVH reactions (>50 mm³) with almost linear relationship up to an inoculum of 10^7 T lymphocytes, beyond which the rise in volume was sharply higher. The ^{125}I-BSA uptake by the local GVH reactions followed a similar rule (Fig. 2). Positive reactions corresponded to $\geq 6,000$ cpm with linear relationship existing up to inocula of 10^7 lymphocytes, beyond which the rise in cpm tended to level off.

DISCUSSION

The results of this study confirm previous reports indicating that the XGVHR is an immunologic phenomenon exerted by competent peripheral-blood T lymphocytes upon recognition of the foreign tissues of the partially immunosuppressed rodent host (4). As a result of this xenogeneic recognition, one observes a modest cell proliferation and a much more pronounced local edema-fluid accumulation. The latter is probably the result of lymphokines released by the T lymphocytes with a marked effect on small blood vessel permeability. Inactivation of the graft cells with mitomycin-C, rendering them incapable of proliferation, results in negative reactions characterized histologically by the appearance of the rodent's polymorphonuclear cells, which are most likely responsible for the "clean-up" of the nonviable graft cells and debris.

The reaction can be consistently and reproducibly elicited with virtually all normal donors' cells, whereas reactivity of cancer patients in this bioassay appears to depend on the stage of their disease. Among those with early stage after resection (NED), 70% are positive, whereas among those with disseminated far-advanced disease, the incidence of positive reaction is only 30%. This finding confirms some previous reports indicating an inverse relationship between general immunocompetence and stage of disease among cancer patients (11-14).

The nature of immune incompetence among cancer patients is not entirely understood. Almost never complete, it probably consists of multiple components, with T lymphocyte malfunc-

tion playing a major role. The capacity of T lymphocytes to exert a GVH reaction in animal models is a net effect resulting from their immune competence and the degree of incompetence or residual rejection capacity of the host (15, 16). This appears to be also true in our system. Thus, biopsies from the macroscopically "negative" reactions observed with cancer patients' cells regularly showed the mononuclear cell infiltration characteristic of the GVH reaction. Nevertheless, the lack of edema-fluid accumulation suggests that lymphokines were either not released or were released but in insufficient amounts by the patients' T lymphocytes upon recognition of the rodent's tissues, indicating immune incompetence. Furthermore, the concomitant presence of the basophils (most likely the rodent's basophils) in those "negative" GVH reactions appears to be analogous to their role in allogeneic rejection (17). Thus, the partially immunosuppressed rodent-host exerts, or at least attempts, a rejection of the graft probably because the graft (cancer patients' lymphocytes) is even more immune incompetent than the host. In contrast, the complete absence of basophilic infiltration by the host in the positive reactions elicited with normal donors' cells attests to the high level of immune competence of the normal graft, which overpowers the residual rejection capacity of the host.

We were able to show that the GVH assay requires a minimum of approximately 4.5×10^6 normal donors' T lymphocytes to induce a positive reaction, the magnitude of which seems to rise linearly with increase in the inoculum up to 10^7 T lymphocytes. It remains to be determined whether the T cell-immune incompetence among cancer patients is a quantitative one resulting only from the reduction in the percentage of T cells encountered among some cancer patients (18), or perhaps a qualitative one that cannot be restored by simply increasing the number of injected T lymphocytes.

Our results with normal donor's subsets and combination of cells suggest that the monocytes may have an immunoregulatory (? suppressor) effect on the normal GVH reaction. However, in cancer patients, elimination of the monocytes that have been incriminated as suppressor cells in some patients (19, 20) was ineffective in the attempt to restore the GVH reaction. It is still possible and remains to be seen whether a subset of T-suppressor cells may be responsible for the immunologic defect observed. Selective elimination (21–23) or perhaps a pharmacologic modulation (24) of such putative T-suppressor cells may have immunotherapeutic implications in the future.

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