CHARACTERIZATION OF CELLS FROM INVADED LYMPH NODES IN PATIENTS WITH SOLID TUMORS

Lymphokine Requirement for Tumor-specific Lymphoproliferative Response

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The cellular responses to tumor cells are mediated by a variety of cell types, such as T cells, NK or lymphokine-activated killer (LAK)[1] cells, and macrophages (1–7). T cells possess the distinctive features of being clonally distributed and of recognizing the antigen on the target cell membrane in association with molecules coded for by genes of the MHC. Three major T lymphocyte subsets (helper, cytotoxic, and suppressor) participate in the response to malignant cells (1–3). The first two cell types may exert a function that is beneficial to the host, whereas suppressor T cells may facilitate tumor growth. The different T cell subsets can be recognized through the expression of distinctive surface markers and can also be tested in a variety of experimental systems. Nevertheless, there has been so far considerable difficulty in elucidating the types of cell interactions that occur during the response to cancer cells. Lack of knowledge in the field has impeded clarification of the mechanisms that lead to a failure of tumor rejection. This has been particularly true for the studies on human tumors, where only in vitro methods could be used. In addition, the prevalent use of peripheral blood as a potential source of tumor-specific T lymphocytes may have contributed to the difficulties encountered in these studies (as discussed in 8–10). In the peripheral blood, T cells with specificity for tumor antigens may be far too diluted, or else their activity may be masked by that of possibly more powerful cytotoxic effector cells such as LAK or NK cells.

In the present investigation, we have explored the possibility that lymph nodes draining the tumor site from patients with cancer of the larynx or of the bladder

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[1] *Abbreviations used in this paper:* GAMIg, goat anti-mouse immunoglobulins antiserum; LAK, lymphokine-activated killer; MLTC, mixed lymphocyte–tumor culture.

could contain tumor-specific T lymphocytes. These experiments were in part suggested by previous observations that these lymph nodes comprise T cells with particular and somewhat unusual surface phenotype (11).

We show that invaded lymph nodes contain T lymphocytes that can be induced to proliferate specifically in response to the patients' malignant cells. A positive response requires, however, that two basic conditions are met, namely that the cultures are supplemented with IL-1 and that a subset of suppressor T cells with a distinctive surface phenotype is removed from the suspensions.

Materials and Methods

Lymph Nodes and Neoplastic Tissues. Pelvic lymph nodes from 18 patients with transitional cell carcinoma of the bladder and cervical lymph nodes from 12 patients with spinocellular carcinoma of the larynx were obtained after informed consent. Each lymph node was divided into two parts for histopathologic evaluation and for in vitro studies. Fragments of tumor tissues were obtained from individual patients under sterile conditions.

Antibodies and Surface Marker Analysis. Anti-T cell mAbs OKT3, OKT4, OKT8 (12) were obtained from Ortho Diagnostic Systems, Milano, Italy. The OKM1 mAb, that recognizes the α chain of the C3bi receptor on monocytes, granulocytes, T cells, and NK cells (13), was also purchased from Ortho Diagnostic Systems. The monocyte-specific Leu-M3 mAb (14) and Leu-11b mAb, which detects the receptor for the Fc portion of IgG on granulocytes and NK cells (15), were purchased from Becton Dickinson Laboratory Systems, Milano, Italy. The B cell–specific B1 mAb (16) was obtained from Coulter-Kontron, Milano, Italy. HNK-1 clone, originally raised by Abo and Balch (17, 18), which produces the Leu-7 mAb, was obtained from the American Type Culture Collection, Rockville, MD. The mAb, which detects subpopulations of NK cells and of T cells, was purified from ascites fluid by filtration on a Ultrogel AcA 34 column. An mAb that reacts with the IL-2 receptor (anti-Tac) (19) was kindly provided by Dr. T. A. Waldmann, National Institutes of Health, Bethesda, MD. PTF 29/12 mAb, an anti-HLA-DR nonpolymorphic determinant (20), and 22.1 mAb, an anti-HLA-A,B,C monomorphic antigen (21), were generous gifts of Dr. G. Damiani, University of Genova.

$F(ab')_2$ fragments of affinity-purified goat anti–mouse Ig (GAMIg) and their FITC-conjugated forms were prepared as previously described (22), and used for cell separation and indirect immunofluorescence analysis, respectively. The polyclonal rabbit anti–human IL-1 antiserum was purchased from Genzyme, Boston, MA.

Surface marker analysis of lymph node cells, or of the cell lines thereof, was carried out by conventional fluorescence microscopy, as described (22). For the quantitative evaluation of Tac antigen expression by cultured lymph node cells, a cytofluorographic analysis was carried out with a FACS (Becton Dickinson Laboratory Systems). 10^6 cells were treated at 4°C for 30 min with anti-Tac mAb (ascites, 1:1,000 final dilution), washed in HBSS, and incubated with $F(ab')_2$ fragments of GAMIg for additional 30 min. An aliquot of freshly isolated cells, stained as above, was fixed in a 1% formaldehyde solution in HBSS, FACS analyzed after 3 d, and compared with cultured cells. Controls included cells treated with an unrelated monoclonal Ig of the same isotype. The data were expressed as a histogram in which the fluorescence intensity (log) was plotted on the abscissa against the relative number of cells on the ordinate. In parallel, volume-distribution histograms, based on forward light scatter, were plotted for each sample.

Cell Separation Procedures. Lymph node fragments were gently dissociated to obtain single-cell suspensions. Mononuclear cells were separated on Ficoll-Hypaque density gradients.

Cells that expressed the Leu-7 marker were fractionated by a direct rosetting technique with ox red cells coated with the purified Leu-7 mAb, as described (23). Cells bearing the OKM1 determinant were separated by indirect rosettes, according to Egeland and Lea (24). Briefly, 2×10^7 cells were resuspended in 400 μl of a 1:10 dilution of the OKM1 mAb in HBSS and kept at 4°C for 30 min. After two washes with cold HBSS, cells were

incubated with a 1% suspension of ox red cells coated with $F(ab')_2$ fragments of GAMIg at 37°C for 1 h. Rosetting cells were then separated by Ficoll-Hypaque density gradients, as described (23). Red cells were lysed by incubating at 4°C for 10 min with a 0.83% NH_4Cl solution.

Peripheral blood mononuclear cells were fractionated into T cells and non-T cells by rosetting with neuraminidase (Sigma Chemical Co., St. Louis, MO)–treated sheep red blood cells, as described (23). Non-T cells were irradiated (3,000 rad) and used as stimulator cells against autologous T cells in a mixed lymphocyte reaction. In all of the above experiments, the rosette-enriched fractions contained >94% rosetting cells.

To isolate tumor cells, fragments of ~1 cm^3 were cut from the tumor mass and mechanically dissociated in the presence of 0.2 mg/ml deoxyribonuclease (Sigma Chemical Co.). To remove large cell aggregates, the suspensions were purified on Ficoll-Hypaque gradients. Tumor cells were separated from tumor-infiltrating lymphocytes by Percoll density gradients, as described by Kedar et al. (25). Briefly, 3×10^7 cells were layered onto a discontinuous gradient of 25, 15, and 10% Percoll in HBSS and centrifuged for 7 min at 25 *g*. The pellet, enriched in tumor cells, was recovered and washed twice; cytocentrifuge smears were prepared to assess the purity of the suspension, which always exceeded 95%. Aliquots of tumor cells were frozen in 90% FCS/10% DMSO (Sigma Chemical Co.) over liquid nitrogen.

Cell Cultures. To test the response of T lymphocytes to tumor cells, various numbers of isolated lymph node cells or fractions thereof were cocultured with irradiated (5,000 rad) autologous or allogeneic tumor cell suspensions in flat-bottom microtiter plates in a total volume of 0.2 ml of RPMI 1640 (Flow Laboratories, Milano, Italy) at 37°C in a humidified atmosphere with 5% CO_2. RPMI 1640 was supplemented with 10% FCS (Gibco, Mascia Brunelli, Milano, Italy), 2 mM L-glutamine (Sigma Chemical Co.), 100 μg/ml streptomycin, 100 IU/ml penicillin (RPMI-FCS). Unless otherwise stated, the cells were cultured for 6 d.

For the autologous mixed lymphocyte reactions, lymph node or peripheral blood cells (or fractions thereof) were cultured with irradiated (3,000 rad) peripheral blood non-T cells, as stimulator cells. 2.5×10^4 responder cells were cultured with an equal number of stimulator cells, using the same conditions as above. Cultures were pulsed with 0.5 μCi of [^{3}H]TdR (sp act 25 Ci/mmol; Amersham, Prodotti Gianni, Milano, Italy), harvested 18 h later, and the radioactivity was determined in a liquid scintillation counter (Beckman Analytical, Milano, Italy).

Statistical evaluation of data was performed by Student's *t* test.

Lymph node cells were stimulated with 1 μg/ml PHA (PHA-P; Wellcome, Pomezia, Italy) or cultured with rIL-2 (25 U/ml), kindly donated by Biogen S.A., Geneva, Switzerland, with purified IL-1 (Genzyme), or human rIL-1-β, a generous gift of Biogen S.A. (0.5–2 U/ml). These experiments were generally carried out by culturing 2.5×10^4 cells/well in 96-well plates. Cell proliferation was assessed as above. When larger cell quantities were needed, the cultures were set up in 24-well plates, at 10^6 cells/ml, in the same conditions as above, or, when indicated, in the presence of rIL-2 or of purified IL-1.

To obtain continuous T cell lines, 2.5×10^5 OKM1$^-$, Leu-7$^-$ cells from metastatic lymph nodes were cocultured in 24-well plates with 2×10^5 irradiated autologous tumor cells in 1 ml of RPMI-FCS, containing 2 U of purified IL-1. After 6 d, the supernatant was removed and replaced with fresh medium containing 25 U/ml rIL-2; at this time, additional tumor cells were added. This procedure was repeated every third day.

To test the proliferative activity of the cell line, 2.5×10^4 cells were cultured for 3 d in the above conditions, with autologous or allogeneic irradiated tumor cells and/or rIL-2. When indicated, the cultures were set up in the presence of PTF 29/12 anti-HLA-DR antibody or 22.1 anti-HLA-A,B,C antibody, 1:1,000 final dilution of ascites fluids.

IL-2 Assay. 4×10^3 murine IL-2-dependent CTLL-2 cells in 100 μl of RPMI-FCS containing 2.5×10^{-5} M 2-ME were incubated with 100 μl of various dilutions of test supernatants, in triplicate, at 37°C. After 24 h, 0.5 μCi of [^{3}H]TdR were added, and incubation was continued for another 4 h. The incorporated radioactivity was assayed as

described above. The IL-2 U/ml were defined as the reciprocal of the supernatant dilution that induced 50% of maximal proliferation of CTLL-2 cells.

IL-1 Assay. IL-1 activity was tested by the thymocyte costimulation assay, as described (23). Briefly, thymuses were surgically removed from C3H/HeJ mice; single-cell suspensions were obtained and thymocytes (1.5×10^6 cells/well) were cultured in triplicate for 72 h in 96-well, flat-bottomed plates, in the presence of 1 μg/ml PHA alone, or PHA plus serial dilutions of the supernatants to be tested. Subsequently, the cells were pulsed with 1 μCi of [^{3}H]TdR for 8 h, harvested, and counted. 1 U of IL-1 was defined as the amount of supernatant capable of doubling the [^{3}H]TdR incorporation by 1.5×10^6 stimulated thymocytes.

Cytotoxicity Assay. Cytotoxic activity was measured in a conventional 4-h assay against ^{51}Cr-labeled cells, as described (23). Briefly, 2×10^6 viable (>80%) tumor cells, or K562 cells, were resuspended in 0.5 ml of RPMI-FCS and incubated with 300 μl of $Na_2{}^{51}CrO_4$ (sp act 200–500 Ci/mmol; NEN, Du Pont Italia, Firenze, Italy) at 37°C for 60 min. The cells were washed three times, kept for an additional 60 min at 37°C, and subsequently washed twice. E/T ratios of 30:1, 10:1, 1:1 were selected. E/T mixtures were incubated for 4 h, were centrifuged, the supernatants were recovered, and their radioactivity was determined. Maximal ^{51}Cr release was determined by lysing the labeled cells with 0.1 N HCl. The percent specific ^{51}Cr release was calculated as: 100 × [(release in presence of effector cells) − (release from labeled cells)]/[(release in presence of HCl) − (release from labeled cells)].

Cold-target inhibition studies were performed by first incubating the effector cells with unlabeled target cells (effector/cold target ratio of 20:1) for 1 h at 37°C, then with ^{51}Cr-labeled cells. The assay was then performed as above.

Results

Response of Lymph Node Cells to Autologous Malignant Cells. In an initial series of experiments, lymphocytes from lymph nodes with and without metastases were tested for their ability to proliferate in response to autologous malignant cells obtained from the primary tumor lesion (mixed lymphocyte–tumor culture [MLTC]). Lymph nodes were subdivided as having or not having metastases according to conventional histopathology. In these experiments, a significant proliferative response was never observed, although a number of variables, such as cell concentration, stimulator/responder cell ratio, duration of coculture period, were tried. Lymph node cells were also studied for their cytotoxic activity against ^{51}Cr-labeled autologous neoplastic cells. Again, a cytotoxic response was never obtained (data not shown).

In principle, the failure to observe a response could be related to any of the following, albeit not mutually exclusive, conditions: (*a*) absence of a tumor antigen(s)–specific lymphocyte population; (*b*) requirement for exogenous lymphokines; (*c*) influence of suppressor mechanisms. These possibilities were investigated as follows.

Cells from both invaded and noninvaded nodes were cultured in the presence of exogenous IL-2, with or without autologous tumor cells. The cells gave a significant response to IL-2, which was not modified by the presence of malignant cells (Table I). The response induced by exogenous IL-2 was not unexpected, because a number of cells stained strongly for Tac antigen (see below and Cozzolino et al. [11]).

Previously, we have shown that metastatic lymph nodes contain a proportion (~20%) of cells that simultaneously express the T3, T8, OKM1, and Leu-7 markers (11). These cells are also present in noninvaded lymph nodes, albeit in

TABLE I

Proliferative Response of Cells from Regional Lymph Nodes to rIL-2

Stimulus	[^{3}H]TdR incorporation*					
	Invaded nodes‡			Noninvaded nodes§		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
	cpm					
Medium alone	250 ± 62	340 ± 87	246 ± 43	405 ± 84	502 ± 48	498 ± 85
Autologous tumor cells	484 ± 58	457 ± 75	358 ± 96	608 ± 101	701 ± 113	655 ± 93
rIL-2‖	5,235 ± 489	6,875 ± 997	4,819 ± 898	4,543 ± 756	3,254 ± 549	2,917 ± 318
Autologous tumor cells + rIL-2‖	5,847 ± 901	6,102 ± 814	5,104 ± 478	3,968 ± 667	3,718 ± 779	3,195 ± 555

* 2.5×10^4 lymph node cells were cultured for 6 d in the presence or absence of 250 irradiated (5,000 rad) autologous tumor cells. Values are expressed as mean ± SD of triplicate cultures.

‡ Data from 3 representative experiments out of 15 performed (8 with bladder, 7 with larynx cancer lymph nodes) are reported.

§ Data from three representative experiments, out of nine performed (five with bladder, four with larynx cancer lymph nodes), are reported.

‖ rIL-2 was used at 25 U/ml final dilution.

TABLE II

Surface Phenotypes of Cells from Metastatic and Nonmetastatic Lymph Nodes

Marker	Percentage of positive cells from:	
	Metastatic nodes (n = 11)	Nonmetastatic nodes (n = 18)
T3	65.5 ± 11.3	54.0 ± 13.9
B1	18.7 ± 3.9	29.4 ± 11.2
Leu-M3	9.1 ± 3.7	7.1 ± 2.9
T4	45.2 ± 10.1	37.2 ± 9.5
T8	39.8 ± 11.5	18.5 ± 6.9
OKM1	27.8 ± 15.3	9.8 ± 3.4
Leu-11	4.9 ± 1.8	6.2 ± 2.7
Leu-7	15.3 ± 7.2	6.9 ± 5.7
HLA-DR	38.9 ± 13.7	42.5 ± 10.2
Tac	21.1 ± 8.2	11.8 ± 4.2

Mean ± SD of pooled data. Lymph nodes from six patients with carcinoma of the bladder and four patients with carcinoma of the larynx were analyzed.

much lower proportions. These data have been confirmed here (Table II and results of double-staining experiments not shown). Because a number of recent observations have indicated that a subset of human suppressor T cells have a $T3^+$, $T8^+$, $OKM1^+$, Leu-7^+ surface phenotype (26, 27), the possibility existed that cells with this phenotype could suppress the in vitro response to malignant cells. Cells from metastatic lymph nodes were depleted of $OKM1^+$, Leu-7^+ cells ($OKM1^-$, Leu-7^- cells) and tested in MLTC. The results are summarized in Table III. $OKM1^-$, Leu-7^- cells were capable of a significant response against malignant cells, provided that exogenous IL-2 was present, i.e., cells cultured with IL-2 and tumor cells proliferated more vigorously than cells cultured with IL-2 alone. Readdition of graded numbers of $OKM1^+$, Leu-7^+ cells (up to the proportions occurring in the unfractionated lymph node) inhibited the response to tumor cells in the presence of IL-2, but did not affect the cell proliferation induced by exogenous IL-2. Taken together, these experiments show that

TABLE III

Proliferative Response of Fractionated Cells from Invaded Lymph Nodes

Stimulus	Numbers of $OKM1^+$, $Leu\text{-}7^+$ cells added	[^{3}H]TdR incorporation by $OKM1^-$, $Leu\text{-}7^-$ cells		
		Exp. 1	Exp. 2	Exp. 3
			cpm	
Medium alone	—	489 ± 27	327 ± 41	521 ± 49
Autologous tumor cells*	—	544 ± 46	389 ± 38	589 ± 23
rIL-2‡	—	7,249 ± 825	8,544 ± 1,154	7,986 ± 1,084
Autologous tumor cells + rIL-2*‡	—	17,589 ± 1,057	18,911 ± 2,185	17,925 ± 1,825
	8.0×10^3	8,648 ± 1,115	8,725 ± 985	9,187 ± 1,101
	4.0×10^3	10,459 ± 988	11,427 ± 1,529	12,185 ± 1,206
	2.0×10^3	13,728 ± 1,224	12,689 ± 1,619	13,888 ± 1,526
	1.0×10^3	15,644 ± 1,229	16,704 ± 1,815	17,119 ± 1,614

Three representative experiments, two with larynx carcinoma and one with bladder carcinoma lymph node cells, out of six performed, are shown. Mean ± SD of triplicate cultures.

* 250 irradiated (5,000 rad) tumor cells were added.

‡ rIL-2 was added at 25 U/ml final dilution.

invaded lymph nodes contain a cell population capable of a proliferative response to tumor cells, which can be detected following removal of suppressor cells and addition of exogenous IL-2.

Cells from lymph node with no metastases failed to respond in MLTC even after removal of the $OKM1^+$, $Leu\text{-}7^+$ cells, perhaps suggesting that cells with specificity for malignant cells were absent (data not shown).

Next we investigated whether or not cells from invaded lymph nodes were restricted to respond to autologous cells or could also respond to allogeneic cells. As shown in Table IV, the proliferation of lymph node cells did not increase above the values induced by IL-2 after addition of allogeneic malignant cells. Failure to respond to allogeneic tumor cells was independent of the histologic type of the tumor cells used for stimulation.

The possibility that $OKM1^-$, $Leu\text{-}7^-$ cells could respond in an autologous MLR was investigated by coculturing these cells with autologous peripheral blood non-T cells. A significant response was never observed, even in the presence of exogenous IL-2, thus indicating that invaded lymph nodes contain a very limited number (if any) of T cells reacting in an autologous MLR. These observations exclude the hypothesis that the antigen(s) recognized by $OKM1^-$, $Leu\text{-}7^-$ cells on malignant cells is represented only by autologous MHC-encoded determinants.

Expression of IL-2 Receptors and Failure of Lymph Node Cells to Produce IL-2 in Autologous MLTC. The problem of whether or not coculture with autologous tumor cells could induce IL-2 receptor expression by $OKM1^-$, $Leu\text{-}7^-$ cells was next addressed. $OKM1^-$, $Leu\text{-}7^-$ cells were cocultured with malignant cells for 3 d and subsequently stained for Tac antigen expression. After coculture with autologous tumor cells, a high proportion of $OKM1^-$, $Leu\text{-}7^-$ cells expressed Tac antigen (Fig. 1*A*). Furthermore, the intensity of staining of the single cells

TABLE IV
Proliferative Response of OKM1⁻, Leu-7⁻ Cells from Metastatic Lymph Nodes to Different Cell Types

Stimulus	[^{3}H]TdR incorporation by OKM1⁻, Leu-7⁻ cells		
	Exp. 1*	Exp. 2*	Exp. 3‡
		cpm	
Medium alone	389 ± 54	527 ± 79	287 ± 44
rIL-2§	6,874 ± 1,657	8,534 ± 1,421	7,741 ± 1,469
Allogeneic tumor cells + rIL-2§,‖			
Bladder tumor I	5,352 ± 538	9,749 ± 1,035	7,397 ± 987
Bladder tumor II	6,835 ± 895	9,562 ± 1,859	8,485 ± 1,024
Larynx tumor I	5,987 ± 1,038	9,001 ± 1,215	6,984 ± 954
Larynx tumor II	7,021 ± 973	8,528 ± 938	7,427 ± 1,045
Autologous tumor cells + rIL-2§,‖	18,348 ± 2,126	16,578 ± 1,741	15,291 ± 1,003
Autologous non-T cells¶	415 ± 171	398 ± 101	361 ± 87
Autologous non-T cells + rIL-2§,¶	7,415 ± 526	8,144 ± 619	6,867 ± 489

Three representative experiments, two with larynx carcinoma and one with bladder carcinoma lymph node cells, out of six performed, are shown. 2.5×10^4 fractionated cells were cultured for 6 h. Mean ± SD of triplicate cultures.

* Fractionated lymph node cells from a larynx cancer patient.

‡ Fractionated lymph node cells from a urinary bladder cancer patient.

§ rIL-2 was added at 25 U/ml final dilution.

‖ 250 irradiated (5,000 rad) autologous or allogeneic tumor cells were added.

¶ 2.5×10^4 irradiated (3,000 rad) autologous peripheral blood non-T cells were added.

increased significantly, as compared to that of Tac$^+$ cells present in the suspension before culture (6–9%) (Fig. 1*A*). Interestingly, the cells that expressed Tac antigen after 3 d belonged to either the T3$^+$, T4$^+$ or to the T3$^+$, T8$^+$ cell subset (data not shown). By contrast, in the suspensions cultured alone or with allogeneic tumor cells there was no increase in the proportion of Tac$^+$ cells (Fig. 1*B*). OKM1$^-$, Leu-7$^-$ cells did not increase in size when cultured with malignant cells. These findings suggest that the encounter with malignant cells provided a sufficient signal for IL-2 receptor expression, but that the cells could not proceed further in their cycle, probably owing to the absence of IL-2.

To study whether or not the responding cells could produce IL-2 in the MLTC, OKM1$^-$, Leu-7$^-$ cells were cultured with malignant cells. IL-2 was measured in the culture supernatants after 3 d. Coculture with either autologous or allogeneic tumor cells failed to induce significant IL-2 production in four consecutive experiments. By contrast, PHA stimulation of the same OKM1$^-$, Leu-7$^-$ cells caused abundant IL-2 release (1–2 U/ml per 10^6 cells).

Characterization of Short-term Lines Derived from MLTC. These experiments were intended to investigate further the fine specificity of OKM1$^-$, Leu-7$^-$ cells and to explore the possibility of obtaining cells with cytotoxic properties against tumor cells. As stated above, no cytolytic cells were detected in freshly prepared suspensions.

OKM1$^-$, Leu-7$^-$ cells were stimulated with IL-2 and autologous malignant cells, and subsequently expanded by further addition of both IL-2 and malignant cells. With this method, five cell lines were obtained. They comprised cells with either T3$^+$, T8$^+$ or T3$^+$, T4$^+$ phenotype, the former being predominant in four

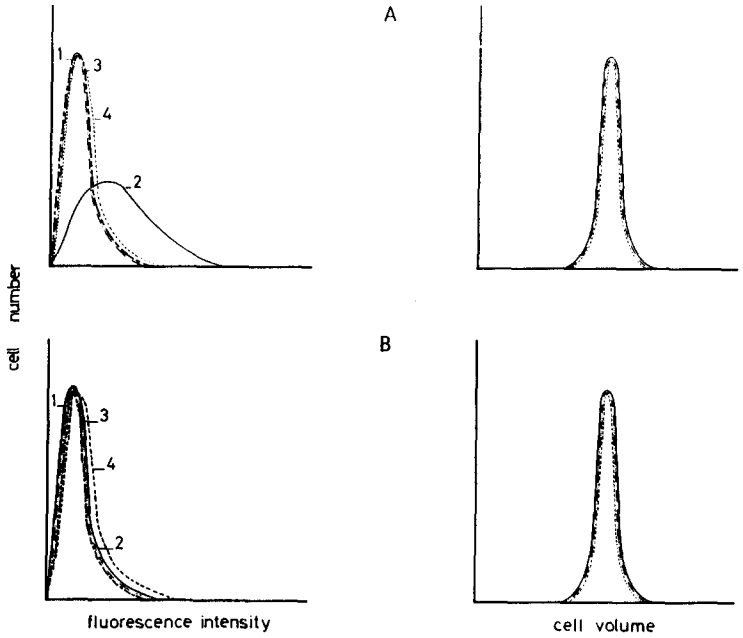

FIGURE 1. FACS analysis of Tac antigen expression by cells from metastatic lymph node. Isolated OKM1$^-$, Leu-7$^-$ cells were cultured for 72 h in the presence (line *2*) or absence (line *3*) of malignant cells and stained with anti-Tac mAb. An aliquot of freshly isolated OKM1$^-$, Leu-7$^-$ cells from the same cell suspension was stained, formaldehyde-fixed, and analyzed after 3 d in comparison with cultured cells (line *4*). Coculture with autologous (*A*), but not with allogeneic (*B*), tumor cells induced Tac antigen expression. In this experiment, representative of five performed, the cells had, at day 0, the following surface phenotype: T3$^+$, 71.3%; T4$^+$, 47.5%; T8$^+$, 21.8; HLA-DR$^+$, 26.3%; Tac$^+$, 7.8%. After 3 d, the phenotype was basically unmodified, except that Tac$^+$ cells were 46.3%. Line *1* shows control with FITC-conjugated goat anti-mouse antibodies (GAMIg) alone. Volume distribution histograms of the same cell populations show no differences in cell size.

of them. The availability of a cell line (FD) that contained predominantly T3$^+$, T8$^+$ cells (i.e., with a cytotoxic cell surface phenotype) allowed to investigate further the question of whether or not a cytotoxic activity could be generated in the system. The T3$^+$, T8$^+$ cell line proliferated specifically in response to autologous but not allogeneic tumor cells in the presence of IL-2 (Table V). Furthermore, the specific proliferative response was abrogated by addition of an anti-HLA-A,B,C mAb and not by anti-HLA-DR mAb. This finding shows restriction of the cells for class I antigens. Interestingly, the treatment with anti-HLA-A,B,C mAb failed to inhibit the response induced by IL-2 alone. The FD cell line showed a weak although definite cytotoxic response against autologous tumor cells. No significant lytic activity was ever observed against three unrelated tumor cells nor to K562 cell line (Fig. 2). The latter control was included to ascertain whether or not the cells possessed an NK-like activity. Specificity for autologous tumor cells was confirmed by cold-target inhibition assays, where autologous but not allogeneic malignant cells inhibited the lytic reaction (Fig. 2).

OKM1$^-$, Leu-7$^-$ Cells Can Be Induced to Mount an MLTC by Addition of IL-1. These experiments were intended to investigate the role possibly played by IL-1 in the MLTC response of OKM1$^-$, Leu-7$^-$ cells. In the absence of IL-1-

TABLE V

Proliferative Activity of Cell Line FD from Metastatic Lymph Node OKM1⁻, Leu-7⁻ Cells

Stimulus‡	[³H]TdR incorporation*
	cpm
Medium alone	1,146 ± 386
Autologous tumor cells	1,427 ± 456
Allogeneic tumor cells I	1,526 ± 712
Allogeneic tumor cells II	1,289 ± 279
rIL-2	13,784 ± 1,126
Autologous tumor cells + rIL-2	21,025 ± 2,284
Autologous tumor cells + rIL-2 + anti-HLA-A,B,C§	11,287 ± 995
Autologous tumor cells + rIL-2 + anti-HLA-DR§	19,857 ± 2,133
Allogeneic tumor cells I + rIL-2	12,825 ± 956
Allogeneic tumor cells II + rIL-2	14,275 ± 1,829

Line FD was raised from a larynx cancer patient and comprised 70–80% cells with a $T3^+$, $T8^+$ surface phenotype. The remaining cells were $T3^+$, $T4^+$.

* Mean ± SD of triplicate cultures. 2.5×10^4 cells were cultured for 3 d.

‡ When indicated, 250 irradiated (5,000 rad) autologous or allogeneic (I from a larynx cancer, II from a urinary bladder cancer) tumor cells and/or rIL-2, 25 U/ml final dilution, were added.

§ Ascites, 1:1,000 final dilution.

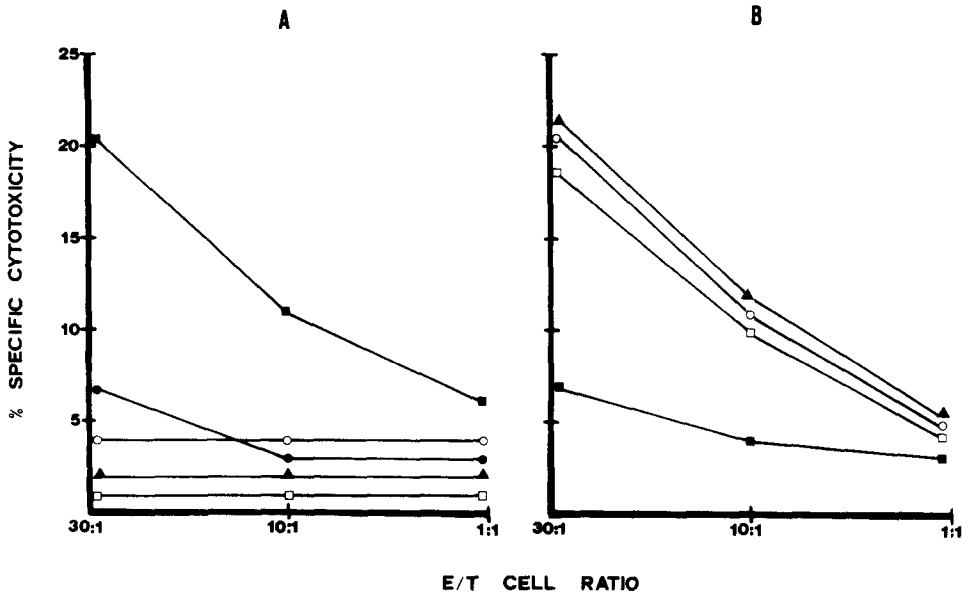

FIGURE 2. Cytotoxic activity of a cell line (FD) derived from OKM1⁻, Leu-7⁻ cells isolated from a metastatic lymph node of a larynx cancer patient. Cells were initially plated in the presence of IL-1 (2 U/ml) and autologous tumor cells, and subsequently expanded in IL-2-containing medium. After 18 d, the cells were tested against ^{51}Cr-labeled autologous tumor cells (■), allogeneic larynx cancer cells (○, ▲), allogeneic bladder cancer cells (□), and K562 cells (●) (*A*). Preincubation with unlabeled autologous (■), but not allogeneic (○, ▲, □), target cells caused a marked reduction in the cytotoxic activity against autologous tumor cells (*B*).

TABLE VI

Proliferative Response and IL-2 Production by OKM1⁻, Leu-7⁻ Cells from Metastatic Lymph Nodes in the Presence of IL-1

Stimulus		Anti-IL-1*	[³H]TdR incorporation‡		IL-2 production§	
IL-1‖	Autologous tumor cells¶		Exp. 1	Exp. 2	Exp. 1	Exp. 2
U/ml			*cpm*		*U/ml*	
1	−	−	4,062 ± 587	3,529 ± 412	0.25	0.25
	+	−	18,443 ± 1,729	15,854 ± 918	1.75	1.50
	+	+	884 ± 312	991 ± 189	—	—
	−	+	419 ± 98	387 ± 121	—	—
0.5	−	−	2,144 ± 648	1,927 ± 344	0.20	0.10
	+	−	16,876 ± 1,385	17,442 ± 995	1.50	1.75
	+	+	745 ± 227	689 ± 212	—	—
	−	+	527 ± 101	581 ± 97	—	—
	Allogeneic tumor cells¶					
1	+	−	4,289 ± 491	3,495 ± 367	0.25	0.20
	+	+	927 ± 115	789 ± 298	—	—
0.5	+	−	2,629 ± 881	2,123 ± 515	0.20	0.10
	+	+	825 ± 191	754 ± 253	—	—
Medium alone	−	−	507 ± 103	485 ± 105	—	—
	+	−	689 ± 81	546 ± 75	—	—

Data from two representative experiments (one with larynx and one with bladder cancer lymph node cells), out of eight performed, are shown.

‡ Mean ± SD of triplicate cultures. 2.5×10^4 OKM1⁻, Leu-7⁻ cells from metastatic lymph nodes were cultured for 6 d.

§ OKM1⁻, Leu-7⁻ cells were cultured at a cell concentration of 10^6 cells/ml. IL-2 activity was measured in the supernatants by the CTLL-2 proliferation assay.

* When indicated, polyclonal rabbit anti-human IL-1 antibodies were added at 1:100 final dilution.

‖ Affinity-purified human monocyte IL-1 was added. Superimposable results were obtained using human rIL-1-β.

¶ When indicated, 250 irradiated (5,000 rad) tumor cells were added.

rich accessory cell supernatants, antigen- or mitogen-activated T cells express IL-2 receptors, but fail to produce IL-2 (28–30). It was therefore possible that the limiting factor for the cells that did not respond in MLTC was represented by the insufficient supply of IL-1. This was also confirmed by the finding that supernatants of 10 different autologous MLTC did not contain detectable IL-1, as assessed in the thymocyte costimulation assay (data not shown). The absence of IL-1 would have in turn impeded both IL-2 production and cell proliferation. To test this hypothesis, OKM1⁻, Leu-7⁻ cells were cultured with different dilutions of purified or recombinant IL-1, in the presence of malignant cells. Representative experiments with optimal doses of IL-1 are shown in Table VI. Addition of IL-1 resulted in cell proliferation, even in the absence of exogenous IL-2. When IL-1 was added, the cells also produced IL-2. Some proliferation was also induced by IL-1 alone, albeit not comparable to that induced by malignant cells plus IL-1. This phenomenon was not attributable to the mitogenic effect of IL-1, possibly contaminated by endotoxin, as T cells purified from peripheral blood were not induced to proliferate by the same IL-1 preparations, under the same experimental conditions (data not shown). Furthermore, stimulation of lymph node cells by IL-1 was inhibitable in the presence of anti-IL-1 antibodies (Table VI). Rather, lymph node cell proliferation in the absence of malignant cells could be related to the presence of a number of cells already

activated by malignant cells in vivo. Alternatively, the $OKM1^-$, $Leu\text{-}7^-$ cell suspension could contain contaminant malignant cells capable of providing the appropriate antigenic stimulus.

Discussion

The present study demonstrates that lymph nodes that drain the tumor site contain cells capable of responding to the malignant cells in vitro. These cells have specificity for autologous but not for allogeneic neoplastic cells, as shown by the tests with a panel of allogeneic malignant cells, and could be detected in a proliferative assay only if two conditions were met: (*a*) exogenous IL-2 must be supplied to sustain the proliferation initiated by coculture with tumor cells, and (*b*) suppressor cells must be removed. Superimposable results were obtained with two different tumor types, a finding that could suggest a common pattern of immune response shared by different neoplasias.

Infiltrated lymph nodes contained lymphocytes with a $T3^+$, $T8^+$, $OKM1^+$, $Leu\text{-}7^+$ surface phenotype that suppressed the response to the malignant cells. The suppressive capacities of $T3^+$, $T8^+$, $OKM1^+$, $Leu\text{-}7^+$ lymphocytes were confirmed by the observation that readdition of such cells to the $OKM1^-$, $Leu\text{-}7^-$ cell suspensions inhibited the response to tumor cells. Suppressor cells have been reported both in mice and humans with malignancies, although different experimental systems have generally been used to detect them (10, 31–34). The mode of action of the suppressor cells described here, together with a characterization of their properties, will be the subject of a separate study.[2] However, it is worth mentioning that the suppression appears to be specific for the response to tumor cells, and is not merely caused by IL-2 consumption by $OKM1^+$, $Leu\text{-}7^+$ cells.

Noninvaded lymph nodes contained neither cells responding to the malignant cells nor suppressor cells. These findings may suggest that cells which respond to the malignant cells remain entrapped in the metastatic lymph nodes and do not recirculate.

The present studies were focused on the mechanisms involved in the stimulation of the cells responding to tumor cells. Coculture with autologous tumor cells induced the expression of IL-2 receptors. This step, however, was not followed by cell proliferation because of lack of IL-2 production in the cultures. Recent work on the multistep process of T cell triggering has shown that resting (or memory) and proliferating T lymphocytes require different signals in order to progress through the cell cycle (28, 29, 35–37). In particular, unlike proliferating cells, resting T lymphocytes require, in addition to the presence of antigen, a signal delivered by IL-1 (28, 29, 38). It is still debated whether or not this signal is necessary for the subsequent expression of IL-2 receptors (39, 40), or whether it induces the production of IL-2 (28, 29). The present data are in agreement with the latter hypothesis. In the absence of detectable IL-1, T cells expressed IL-2 receptors after antigen stimulation, but began to produce IL-2 only after the addition of exogenous IL-1. While the absence of IL-1 production

[2] Cozzolino, F., M. Torcia, A. M. Carossino, R. Giordani, C. Selli, G. Talini, E. Reali, A. Novelli, V. Pistoia, and M. Ferrarini. Functional analysis of suppressor cells from regional lymph nodes of cancer patients. Manuscript in preparation.

explains the failure of T cells to produce IL-2 in the MLTC, there are no clear explanations for the lack of IL-1 production after stimulation with malignant cells. One possible interpretation could be that macrophages or other accessory cells committed to IL-1 production are not induced to do so solely by the presence of (or encounter with) neoplastic cells. An alternative and not mutually exclusive explanation may be that T cells have to activate accessory cells to release IL-1. This activation may be mediated, for example, through the production of IFN-γ (41, 42). The recognition of tumor cells may not be a sufficient signal for inducing the release of this lymphokine. The possibility also exists that macrophages from the metastatic lymph nodes have an impaired capacity of producing IL-1, as reported in other experimental systems (43). Finally, the possible presence of IL-1 inhibitors has to be considered (44). All of the above hypotheses are compatible with the observation that OKM1$^-$, Leu-7$^-$ cells respond to PHA. The response to this mitogen is less dependent on accessory cells, and PHA per se induces abundant IL-1 release by macrophages (45).

The short-term lines derived from the cells responding to tumor cells required the constant presence of IL-2 for antigen-induced proliferation. This observation raises the question of whether or not they represent a special T cell subset. However, so far, the surface marker analysis has not revealed particular differences between cells responding to tumor cells and those directed to other types of antigen. Furthermore, cells that are dependent on constant stimulation with both antigen and IL-2 for growth have been described also in different experimental systems (46–49).

T cells isolated from lymph nodes were not cytotoxic for the malignant cells when tested immediately after isolation. Cytotoxic T cells were, however, detected in one of the cell lines obtained. The availability of this cell line provided a further opportunity for testing both the antigen specificity and the restriction of the responding cells. Again, these cells were induced to proliferate only by the autologous malignant cells. Furthermore, their proliferative response was inhibited by an anti-HLA-A,B,C mAb, as could be expected for class I–restricted cytolytic T cells. Finally, the lytic activity was exerted only and specifically on the autologous malignant targets. Taken together, these data indicate that lymph nodes from patients with malignancies contain cytotoxic T cell precursors, which may be usable in novel therapeutical approaches.

Summary

The specific immune response against the malignant cells was investigated in patients with urinary bladder or larynx cancer. Lymphocytes from lymph nodes that drain the tumor site were tested for their proliferative and cytotoxic capacities against autologous malignant cells isolated from the primary tumor. In no occasion was a proliferative or a cytotoxic response observed. However, when the lymph node cell suspensions were depleted of cells expressing both OKM1 and Leu-7 markers by rosetting with the appropriate mAbs, a proliferative response could be observed. The lymphocytes responded to autologous tumor cells only if IL-2 was added to the cultures. IL-2 alone induced some cell proliferation, which was not, however, comparable to that observed in response to both IL-2 and tumor cells. A panel of allogeneic tumor cells consistently failed

to stimulate OKM1$^-$, Leu-7$^-$ cells in vitro. Response to autologous tumor cells was not caused by HLA-encoded molecules, as occurs in the autologous mixed lymphocyte reaction, since OKM1$^-$, Leu-7$^-$ cells failed to be stimulated by autologous non-T cells. A proliferative response was observed only with cells from lymph nodes that had been classified as invaded by malignant cells according to histopathologic criteria. Cells from noninvaded lymph nodes consistently failed to respond.

Cells stimulated with autologous tumor cells could be expanded in short-term lines by continuous addition of IL-2 and malignant cells. One of these lines, which comprised mainly T8$^+$ cells, was stimulated to proliferate only by autologous tumor cells, and its proliferative response was inhibitable by anti–class I and not by anti–class II mAbs. This line showed lytic capacities against autologous malignant targets, while it was inefficient against all of the other allogeneic cells tested.

In another set of experiments, the mechanisms whereby exogenous IL-2 had to be added to the cultures to sustain a proliferative response against neoplastic cells were investigated. When cocultured with autologous malignant cells, OKM1$^-$, Leu-7$^-$ lymphocytes expressed IL-2 receptors, as could be assessed by anti-Tac fluorescent staining. Under these culture conditions, these cells did not produce IL-2, and no proliferation was observed. Addition of purified IL-1 to the cultures induced IL-2 production and cell proliferation.

It is concluded that metastatic lymph nodes contain a T cell population that can be detected in a proliferative assay when both suppressor cells are removed and the appropriate molecular signals are supplied.

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