ORIGINAL ARTICLE

Cell-mediated Cytotoxicity of Tumor Infiltrating Lymphocytes on Rat Bladder Cancer

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Abstract. Tumor-infiltrating lymphocytes (TIL) were tested for cytotoxicity against autologous tumor cells in a study utilizing a chemically induced cancer of the bladder (transitional cell carcinoma), BC-47, in inbred ACI/N rats. From tumors grown after subcutaneous implantation of BC-47 in the rats TIL were separated by density gradient centrifugation and incubated in plastic dishes for separation of non-adherent from adherent cells. The non-adherent cells were further fractionated into T and B cells by the panning method using anti-rat F(ab')₂ antibody. The cell fractions were each added to BC-47 in culture to be assessed for antitumor effect by the crystal violet dye exclusion method and ³H-thymidine incorporation inhibition assay. Peripheral blood mononuclear cells (PBMC) were also tested as described above. TIL expressed significantly higher cytotoxicity against BC-47 with the mean % cytotoxicity of 56.6 ± 5.6% and 87.5 ± 7.1% at E/T ratios of 10:1 and 20:1, respectively, as compared to PBMC (9.9 ± 5.0% at E/T 10:1) (P<0.001). The adherent cells, B and T cell fractions showed respective % cytotoxicity of 92.4 ± 2.8%, 57.9 ± 10.6% and 9.9 ± 7.8% at an E/T ratio of 10:1. TIL pretreated with IFN or rIL-2 for 24 or 48 hours did not exhibit any noticeably enhanced antitumor activity at an E/T ratio of 5:1. Prevention of direct contact of BC-47 cells and TIL by an interposed Millipore membrane (0.45 μm) resulted in an unequivocal reduction of antitumor effect. This finding clearly indicates that the direct contact with BC-47 cells and TIL is necessary for TIL to manifest their cytotoxicity against the autologous tumor cells, suggesting that TIL exhibit autologous tumor killing activity through cell-mediated cytotoxicity. (Keio J Med 41 (3): 146–153, September 1992)

Key words: TIL, BC-47, cell-mediated cytotoxicity

Introduction

A variety of studies have so far been conducted on immunocytes that participate directly in tumor cell destruction and are responsible for antitumor immunity in cancer-bearing hosts. While, among the immunologically competent cells, reported studies on monocytes^{1–7}/macrophages^{8–12} and natural killer (NK) cells^{13,14} are particularly numerous, lymphokine-activated killer (LAK) cells, NK cells and cytotoxic T lymphocytes (CTL) have become the subjects of a number of intensive studies since Rosenberg *et al*¹⁵ advocated the possible importance of LAK cells activated by interleukin-2 (IL-2) in tumor immunity.^{16–22} Many of these studies, however, pertained to systemic immune reactions.

One can readily assume that, in cancer-bearing hosts, a host immunosurveillance mechanism will become operative first at the site of tumor growth and it seems apparent, therefore, that to elucidate the local immunosurveillance mechanism is essential for better understanding of the host immunosurveillance mechanism at large. Tumor-infiltrating lymphocytes (TIL) have recently been attracting attention because of their putative role of great importance in local immunosurveillance, although this particular population of lymphocytes remain yet to be fully characterized. The present experimental study represents our attempt to elucidate the mechanism whereby TIL express their cytotoxicity against autologous tumor cells, using a bladder cancer BC-47 induced in inbred ACI/N rats, a malignant tumor cell line that has

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the advantage of being able to be maintained in subcultures both *in vivo* and *in vitro* concurrently and, accordingly, well serves the purposes of the study.

Materials and Methods

Experimental animals

Female inbred ACI/N rats at 6 to 8 weeks of age were utilized.

Target cells

A bladder cancer cell line (transitional cell carcinoma) BC-47^{23,24} induced chemically by exposure to N-butyl-N-butanol (4) nitrosamine (BBN) in inbred ACI/N rats was used as target cells.

Culture medium

RPMI-1640 (GIBCO, Grand Island, New York) containing 10% fetal bovine serum (FBS, Flow Lab, Virginia, USA) supplemented with 10^5 U/L penicillin G, 100 mg/L streptomycin, 2 g/L sodium bicarbonate and 300 mg/L L-glutamine was used as tissue culture medium.

Preparation of tumor-infiltrating lymphocytes (TIL) and peripheral blood mononuclear cells (PBMC)

TIL were prepared from tumor tissue as described by Moy *et al.*²⁵

BC-47, 5×10^6 cells/0.2 ml, were implanted into the subcutaneous tissue of ACI/N rats and as soon as a tumor mass of 2.5 cm longer diameter was formed, the tumor was excised aseptically and, upon removal of normal and necrotized tissues, was minced in a Petri dish containing 5 ml RPMI-1640 supplemented with 10% FBS, 10 mM HEPES buffer and antibiotics, and filtered through a wire mesh. The filtrate was centrifuged at $400 \times g$ for 10 minutes and the supernatant discarded. After repeating this washing procedure three times the pellet was suspended in 10 ml RPMI-1640 supplemented with 10% FBS. The cell suspension was layered on 10 ml Ficoll-Hypaque gradients (Lymphoprep liquid, Daiichi Kagaku; specific gravity 1.077) and centrifuged at $400 \times g$ for 30 minutes at 4°C (KR/180 B, KUBOTA). Pelleted cells in the white cuff layer were collected, resuspended in 10 ml RPMI-1640 supplemented with 10% FBS and centrifuged at $400 \times g$ for 10 minutes. TIL were obtained by repeating this washing procedure twice. Peripheral blood mononuclear cells were prepared in the same manner as above from blood samples taken via the inferior vena cava concurrently with removal of tumor masses.

Cell separation

Isolated TIL were incubated in plastic dishes pre-coated with FBS to separate non-adherent cells from adherent cells. The non-adherent cells were further sorted into T cell fraction and B cell fraction by the panning method²⁶ using anti-rat F(ab')₂ goat antibody (Cooper Biomedical, Inc, Malvern) (Fig 1). Separation of classes of PBMC was also carried out in a similar manner.

Morphologic examination (Giemsa-stained cytocentrifuge preparation¹³)

TIL of 2×10^5 cells suspended in 0.2 ml culture medium were spread on a slide glass and centrifuged in a Cytospin centrifuge (Shandon Southern Instrument Inc, Bewickley, PA, USA) at 900 rpm for 7 minutes. Then, the cells were fixed in methanol for 10 minutes in the atmosphere and stained with a 10% dilution of Giemsa stain in buffer solution of pH 7.4 for 25 minutes. At least 200 Giemsa-stained TIL per preparation were analyzed under an oil-immersion microscope.

Fig 1 Cell separation. Isolated TIL were incubated in plastic dishes precoated with FBS to separate non-adherent cells from adherent cells. The non-adherent cells were further sorted into T cell fraction and B cell fraction by the panning method using anti-rat F(ab')₂ goat antibody.

Mixed culture with interferon or interleukin-2

Cell separations containing 1×10^6 TIL and 100, 1,000 and 10,000 IU/ml of rat interferon (IFN, Lee Biomolecular Research Laboratories, Inc, USA) or 1,000, 2,000 and 3,000 IU/ml of recombinant interleukin-2 (rIL-2, Shionogi Pharmaceutical Co) were prepared and incubated in a culture flask (Falcon 3013 Becton, Dickinson Lab, Oxnard, USA) for 24 or 48 hours in atmosphere of 5%CO₂ in air at 37°C.

Cytotoxicity assay (Crystal violet dye exclusion method)

BC-47 target cells were placed in wells of 96-well microtiter plates (Falcon 3072 Becton, Dickinson Lab, Oxnard, USA) at a concentration of 1×10^3 cells/0.1 ml/well in triplicate. Then, the mononuclear cells were added in sufficient numbers to give effector/target (E/T) ratios of 1:1, 5:1, 10:1 and 20:1. The mixtures were incubated for 24, 48 and 72 hours in a 5%CO₂ air incubator at 37°C. Thereupon, the culture medium was discarded and cells were stained with 0.2% crystal violet in 2% ethanol, washed in PBS, dried and measured for absorbance with a microplate photometer (CMPT-02, CORONA, Ibaraki, Japan) using a 414nm filter. The antitumor effect was calculated from the following formula²⁷:

$$\% \text{ cytotoxicity} = 100 \times (1 - T/C)$$

T = absorbance of experimental group

C = absorbance of control group

All experiments were carried out in triplicate.

Cytostatic assay (³H-thymidine incorporation inhibition assay¹)

Mixtures of BC-47 cells and TIL were incubated for 24, 48 and 72 hours in the same manner as described above. Four hours before termination of cultivation 1 μ Ci ³H-thymidine was added to each well and upon termination of cultivation the cells were recovered by means of a harvester Mark II (Labo Mash, Labo Science Co) and measured for radioactivity in a liquid scintillation counter. The antitumor effect was calculated from the following formula¹:

$$\% \text{ inhibition of } ^3\text{H-thymidine uptake} = \frac{(\text{cpm of T}) - (\text{cpm of T and E})}{(\text{cpm of T})} \times 100$$

T: target cells E: effector cells

Direct target cell-TIL contact prevention experiment

BC-47 cells and TIL (at an E/T ratio of 10:1) were

Fig 2 Direct target cell-TIL contact prevention experiment. BC-47 cells and TIL (at an E/T ratio of 10:1) were incubated for 3 days in wells of 24-well microtiter plates while being prevented from coming in contact with each other by use of Millicell-HA of 0.45 μ m pore size.

incubated for 3 days in wells of 24-well microtiter plates (NUNCNCLON, NUNC Co, Denmark) while being prevented from coming in contact with each other by use of Millicell-HA of 0.45 μ m pore size (Millipore, USA). Then, after removing dead cells as detected by the trypan blue dye exclusion method, target cell numbers were counted with a hemocytometer, from which to estimate antitumor effect (Fig 2).

Results

Separation of TIL and PBMC

Tumor specimen weights were 4.9 ± 0.6 g (mean $\pm$ SD) and then $5.5 \pm 2.4 \times 10^6$ TIL could be collected from tumor masses, while blood sample volumes were

3.0 ± 0.5 ml and then $1.35 \pm 0.15 \times 10^6$ PBMC could be collected from donor blood. TIL were found to be more than 90% viable according to the trypan blue dye exclusion method and contain $7.1 \pm 0.9\%$ contaminating tumor cells. The percentile proportions of individual fractions of TIL were: adherent cells $30.0 \pm 6.8\%$, T cell fraction $53.3 \pm 10.3\%$ and B cell fraction $16.6 \pm 4.4\%$. As the yield of TIL increased, the relative proportion of T cell fraction increased and those of adherent cells and B cell fraction decreased (data not shown).

Morphologic examination

A microscopic analysis of TIL revealed $20.5 \pm 3.6\%$ of Giemsa-stained mononuclear cells to be macrophages, which, moreover, accounted for $86.3 \pm 2.9\%$ of adherent cells, $14.2 \pm 6.7\%$ of B cell fraction and $4.6 \pm 4.1\%$ of T cell fraction.

Antitumor effect of TIL

Cytotoxic effect (crystal violet dye exclusion method): The cytotoxic effect of TIL was assessed after 1, 2 and 3 days of incubation. Percent cytotoxicities observed were $7.2 \pm 5.4\%$, $16.0 \pm 10.8\%$ and $16.3 \pm 12.8\%$, respectively, at E/T ratio of 1:1, $10.7 \pm 4.4\%$, $33.1 \pm 13.7\%$ and $32.3 \pm 2.9\%$, respectively, at 5:1, $36.4 \pm 18.1\%$, $41.1 \pm 7.1\%$ and $56.6 \pm 5.6\%$, respectively, at 10:1, $59.0 \pm 8.8\%$, $52.0 \pm 0.9\%$ and $87.5 \pm 7.1\%$, respectively, at 20:1 (Fig 3). Thus, the cytotoxic effect was most striking at an E/T ratio of 20:1.

Cytostatic effect (^3H -thymidine incorporation inhibition assay): Similarly, this assay was performed after 1, 2 and 3 days of incubation. Percent inhibition of ^3H -thymidine uptake observed were $54.0 \pm 6.4\%$, $35.3 \pm 6.4\%$ and $28.8 \pm 4.8\%$, respectively, at an E/T

ratio of 1:1; $71.6 \pm 2.3\%$, $71.6 \pm 2.1\%$ and $47.9 \pm 3.5\%$, respectively, at 5:1; $81.8 \pm 4.7\%$, $71.0 \pm 1.8\%$ and $72.3 \pm 2.0\%$, respectively, at 10:1 and $92.6 \pm 2.8\%$, $90.4 \pm 2.2\%$ and $93.4 \pm 1.4\%$, respectively, at 20:1 (Fig 4).

Antitumor effect of individual fractions of TIL (crystal violet dye exclusion method)

The antitumor effect of each individual fraction of TIL was evaluated at an E/T ratio of 10:1 after 1, 2 and 3 days of incubation. The observed % cytotoxicity values were $50.0 \pm 7.2\%$, $68.2 \pm 3.4\%$ and $92.4 \pm 1.8\%$, respectively, for adherent cells, $38.9 \pm 8.3\%$, $57.0 \pm 9.5\%$ and $57.9 \pm 10.6\%$, respectively, for B cell fraction, and $37.8 \pm 4.2\%$, $15.2 \pm 0.4\%$ and $9.9 \pm 7.8\%$, respectively, for T cell fraction (Fig 5). The adherent cells were thus shown to be most potent in antitumor effect.

Fig 3 Kinetics of cytotoxic effect of TIL. Percent cytotoxicities were assessed by the crystal violet dye exclusion method.

Fig 4 Kinetics of cytostatic effect of TIL. Percent inhibition of ^3H -thymidine uptake were assessed by ^3H -thymidine incorporation inhibition assay.

Fig 5 Kinetics of cytotoxic effect of individual fractions of TIL. Percent cytotoxicities were assessed by the crystal violet dye exclusion method. E/T ratio = 10:1.

Antitumor effect of PBMC (crystal violet dye exclusion method)

PBMC were also assessed for antitumor activity after 1, 2 and 3 days of incubation. Percent cytotoxicity values thus found were $4.8 \pm 8.2\%$, $9.3 \pm 2.7\%$ and $13.1 \pm 7.7\%$, respectively, at an E/T ratio of 1:1, $7.1 \pm 7.1\%$, $26.2 \pm 3.6\%$ and $9.8 \pm 7.2\%$, respectively, at 5:1; $6.9 \pm 7.0\%$, $29.6 \pm 8.5\%$ and $9.9 \pm 5.0\%$, respectively, at 10:1 (Fig 6). The antitumor effect of PBMC was thus apparently less pronounced than that of TIL ($p < 0.001$).

Direct target cell-TIL contact prevention experiment (trypan blue dye exclusion method with a hemocytometer)

TIL were assessed for antitumor effect after being incubated for 3 days with BC-47 at an E/T ratio of 10:1 but while prevented from coming in contact with the target cells. Percent cytotoxicities observed with adherent

Fig 6 Kinetics of cytotoxic effect of PBMC. Percent cytotoxicities were assessed by the crystal violet dye exclusion method.

Table 1 Cytotoxic Effect of TIL Prevented from Direct Contact with BC-47 Cells

	% Cytotoxicity (Mean $\pm$ SD)	
	Without Membrane (Without Prevention from Direct Contact)	With Membrane (With Prevention from Direct Contact)
Adherent Cells	$88.7 \pm 4.1\%*$	$17.2 \pm 2.4\%*$
Non-adherent Cells	$57.4 \pm 4.9\%**$	$0.9 \pm 0.7\%**$

BC-47 cells and TIL (at an E/T ratio of 10:1) were incubated for 3 days while being prevented from coming in contact with each other by use of Millicell-HA of 0.45 μ m pore size.

Percent cytotoxicities were assessed by the trypan blue dye exclusion method with a hemocytometer.

*: $p < 0.001$, **: $p < 0.001$

cells were $17.2 \pm 2.4\%$ with as against $88.7 \pm 4.1\%$ without prevented contact with the target cells, while the corresponding values for non-adherent cells were $0.9 \pm 0.7\%$ vs $57.4 \pm 4.9\%$ (Table 1). It became thus obvious that prevention of the effector cells from direct contact with the target cells during incubation period resulted in an unequivocal diminution of antitumor effect.

Antitumor effect of IFN- or rIL-2-treated TIL (crystal violet dye exclusion method)

TIL pretreated with IFN or rIL-2 for 24 or 48 hours

Fig 7 Kinetics of cytotoxic effect of TIL pretreated with IFN or rIL-2 for 24 hours. Cell suspensions containing 1×10^6 TIL and 100, 1,000 and 10,000 IU/ml of rat IFN or 3,000 IU/ml of rIL-2 were incubated in a culture flask for 24 hours in atmosphere of 5% CO_2 in air at 37°C. Percent cytotoxicities were assessed by the crystal violet dye exclusion method. E/T ratio = 5:1.

Fig 8 Kinetics of cytotoxic effect of TIL pretreated with IFN or rIL-2 for 48 hours. Cell suspensions containing 1×10^6 TIL and 100, 1,000 and 10,000 IU/ml of rat IFN or 3,000 IU/ml of rIL-2 were incubated in a culture flask for 48 hours in atmosphere of 5% CO_2 in air at 37°C. Percent cytotoxicities were assessed by the crystal violet dye exclusion method. E/T ratio = 5:1.

were tested for antitumor activity (Figs 7 and 8). These IFN- or rIL-2-pretreated effector cells did not exhibit any noticeably enhanced antitumor activity at an E/T ratio of 5:1. Pretreatment of TIL with rIL-2 at 1,000 or 2,000 IU/ml (data not shown in Figs 7 and 8) did not result in any overt augmentation of antitumor effect as was the case with TIL treated at 3,000 IU/ml.

Discussion

Concerning tumor-infiltrating lymphocytes in bladder cancer tissues, Tsujihashi *et al*²⁸ reported that whereas B cells were abundant in inflamed bladder tissues, T cells were predominant lymphocytes infiltrating bladder tumors and tended to be more so in malignant tumors of lower depth of invasion. They stated further that NK cells, though less abundant than T cells, showed a more marked tendency to infiltrate neoplastic tissues than the latter cells and were found particularly numerous in malignancies of low depth of invasion, while macrophages were present, though quite limited in number, in 13 out of 25 human bladder cancer cases. According to Moy and his associates,²⁵ by contrast, TIL prepared from human pulmonary tumors after mechanical disaggregation contained an average of $21.2 \pm 5.7\%$ macrophages/monocytes. On the other hand, TIL samples prepared from rat bladder cancers in the present study comprised 53.3% T cell fraction, 16.6% B cell fraction and 30.0% adherent cells, while morphological examination of Giemsa-stained smears revealed mononuclear cells to contain 20.5% macrophages. As a high percentage of rat bladder tumor masses of larger than 4 cm in diameter had central necrosis with a consequent diminution of TIL yield (data not shown), autologous tumors in rats were excised as soon as they grew to attain a longer diameter of 2.5 cm. Moy *et al*²⁵ also recognized significant infiltration of TIL in firm or fibrous tumors than tumors having central necrosis. This was interpreted as indicating that the distribution of TIL varies with different extents of tumor growth and is not related directly to the capability of tumor immunity.

In many reported studies, TIL have been tested for short-term cytotoxicity against freshly isolated autologous tumor cells using ⁵¹Cr release assay. Since, however, it seems very much likely that in actual tumor lesions, TIL fully express cytotoxic activity on prolonged contact with tumor cells, it is felt necessary to investigate the long-term cytotoxicity of TIL also. An attempt was made in the present study, therefore, to test TIL for antitumor activity on a relatively long-term basis by use of the crystal violet dye exclusion method and ³H-thymidine incorporation inhibition assay. Evidence has been accumulated that TIL have a profound antitumor effect.^{29–34} However, available evidence also indicates that TIL are variable in the biologic nature of target

tumor cells. Thus, some TIL specimens were reported to be highly cytotoxic for both autologous and allogeneic tumor cells,^{29,30} while others were preferentially more cytotoxic to autologous tumor cells^{31–33} or cytotoxic exclusively to autologous^{32,33} or allogeneic tumor cells.³³ Furthermore, TIL have been shown to have higher cytotoxic activity than peripheral blood lymphocytes (PBL).^{29–32} Also in the present study, TIL were demonstrated to have a pronounced antitumor effect on autologous tumors and to be much higher than PBMC in cytotoxic activity against those tumor cells. Among cell fractions that were identified as expressing cytotoxicity through cell separation, adherent cells were shown to be strongest in antitumor effect. Monocytes^{1–7}/macrophages^{8–12} have been reported to have pronounced cytotoxic activities. Nakamura¹ found Bacillus Calmette-Guérin (BCG)-enhanced monocytes to produce a strong cytostatic effect on a bladder tumor cell line, KU-7. Wiltrout *et al*¹⁰ also reported activated peritoneal macrophages to exhibit a strong cytotoxic activity. One can surmise that the pronounced antitumor effect observed with adherent cells in the present study was produced by tumor-infiltrating macrophages the migration of which had been suppressed by macrophage migration inhibition factor (MIF) and which had been rendered active by macrophage-activating factor (MAF). In view of the fact that the B cell fraction contained 14.2% macrophages, it would also seem reasonable to construe the observed antitumor effect of this cell fraction as due to the tumoricidal capabilities of contaminating macrophages.

According to a report by Itoh *et al*,³² TIL that were isolated from human metastatic melanoma and subsequently cultured in the presence of rIL-2 (rIL-2-activated TIL) expressed high cytotoxicity against allogeneic tumors, being comprised predominantly of CD8⁺ T cells (CTL). In contrast, freshly isolated TIL comprised 80% CD3⁺ T cells and less than 2% CD16⁺ NK cells and did not express any noticeable cytotoxicity against autologous tumors. On the other hand, TIL freshly isolated from human renal cell carcinoma contained 75% CD3⁺ T cells and 5% CD16⁺ NK cells, while the TIL, after being activated by rIL-2, consisted of 75% CD3⁺ T cells and 25% CD16⁺ NK cells and exhibited marked cytotoxicities both against autologous tumors and allogeneic tumors, with the NK cells being recognized to show more profound cytotoxicity than the T cells. While the cell type that is responsible for cytotoxicity of TIL may vary with different tumor sources, as is apparent from the Itoh *et al*'s report, CTL or NK cells or both have been identified as such in many of other studies.^{29–33} Insofar as the present study is concerned, the cytotoxicity of T cell fraction against autologous tumor was low. If CTL and/or NK cells are really responsible for TIL cytotoxicity, T cell fraction would then be anticipated to show by far the highest cytotoxicity among other cell fractions. It

should be noted, however, that most previous studies on TIL²⁹⁻³⁴ concerned the cytotoxicity of rIL-2-stimulated and activated TIL and differ essentially from our present study which relates to the T cell fraction of freshly isolated TIL. Moreover, it is a likely possibility that freshly isolated T cell fraction from a bladder tumor BC-47 in ACI/N rats is not cytotoxic for autologous tumors, as were freshly isolated melanoma TIL to autologous tumors in the aforementioned Itoh *et al*'s study. One can assume that this explains the reason why the T cell fraction showed lower cytotoxicity against autologous tumors than expected. Weber and associates,³⁴ in their experimental study on murine B16BL6 melanoma, reported that whereas TIL isolated from class I major histocompatibility complex-deficient tumors proliferated only in the presence of high-dose rIL-2 (1,000 U/ml) but not all with low-dose rIL-2 (20 U/ml) and did not express cytotoxic effects both *in vitro* and *in vivo*, those from class I MHC-expressing tumor cells did proliferate both with low-dose and with high-dose rIL-2 and expressed strong cytotoxic effects both *in vitro* and *in vivo*. In the present study, attempts to proliferate and activate rat TIL by IFN or rIL-2 of varying concentrations were unsuccessful in augmenting their cytotoxic capacity to any noticeable extent. This might be interpreted as suggesting that TIL isolated from rat bladder cancer BC-47 consist of cell types that are not susceptible to activation by rIL-2.

The direct target cell-TIL contact prevention experiment carried out in an effort to elucidate the mechanism for expression of TIL cytotoxicity demonstrated an unequivocal diminution of antitumor effect to result from the prevention of direct contact of the effector with target cells during incubation. The results of this experiment in the present study led us to speculate that the TIL require direct contact with the target cells for expression of cytotoxicity against those autologous tumor cells and that TIL cytotoxicity thus expressed is more cell-mediated than cytokine-mediated in nature.

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