LYMPHOID CELLS MEDIATING TUMOR-SPECIFIC CYTOTOXICITY TO CARCINOMA OF THE URINARY BLADDER

Separation of the Effector Population using a Surface Marker*

By C. O'TOOLE, V. STEJSKAL, P. PERLMANN and M. KARLSSON

(From the Department of Immunology, Wenner-Gren Institute, Norrtullsgatan 16, S-113 45 Stockholm, Sweden)

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In vivo immunization in the mouse with allogeneic or syngeneic malignant cells results in the development of thymus-derived (T)[1] cytotoxic effector cells (1). Virus-induced murine tumors stimulate both thymus-dependent and thymus-independent effector cells during different phases of tumor development (2, 3). However, injection of xenogeneic cells into the rat has been shown to raise a population of thymus-independent effector cells (4).

In vitro lymphocytes from nonimmunized donors have been shown to be rendered cytotoxic in the presence of target cell-specific antibody (4, 5). The cells participating in this type of reaction appear to be members of the thymus-independent population. Similarly non-T lymphoid cells from animals immunized with soluble antigens are cytotoxic in vitro towards target cells coated with the specific antigen (6).

Patients with transitional cell carcinoma (TCC) of the urinary bladder have been shown to possess cytotoxic effector cells in the blood, with specific reactivity for tumor cells of relevant origin (7–11). Removal of cells carrying Fc receptors and/or surface immunoglobulin (Ig), from such cytotoxic lymphocyte preparations results in a loss of existing activity (12).

The data to be presented here show that after removal of the cells forming spontaneous rosettes with sheep erythrocytes (E) from cytotoxic populations, the tumor-specific activity lies in the nonrosette-forming cell fraction. This procedure is known to deplete blood lymphocytes of T cells (13, 14) and to concentrate the cell fraction which is involved in antibody-mediated cytotoxicity (15).

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[1] *Abbreviations used in this paper:* E, sheep erythrocytes; EA, erythrocytes coated with IgM antibody without complement; EAC, erythrocytes coated with IgM antibody plus complement; FCS, fetal calf serum; FI, Ficoll-Isopaque solution; MEL-1, primary culture of metastatic melanoma; MGG, May-Grünwald Giemsa stain; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; T, thymus derived; TCC, transitional cell carcinoma; and TH, tris-buffered Hanks' solution.

Materials and Methods

In eight separate experiments lymphoid cells from eight patients with clinically verified TCC were compared with those of eight healthy controls. TCC was staged and graded as described previously (9). The clinical situation of the patients is summarized in Table III.

Preparation of Effector Cells.—Preparation of effector cells was as described previously (9, 16). About 30 ml of defibrinated blood were obtained from each donor. The bulk of erythrocytes were sedimented on gelatin (17). The leukocyte-plasma supernatant was then incubated on a nylon wool column (18) to remove adherent cells. The cells eluted from the column were then washed three times with Tris-buffered Hanks's solution (TH) containing 2.5% fetal calf serum (FCS) (heat-inactivated 60 min) and counted in Turk's stain.

Preparation and Fractionation of E Rosette-Forming Cells.—A population of human lymphocytes forming direct rosettes with sheep erythrocytes have the characteristics of T cells (13, 19, 20). E rosettes were formed according to Yata's method (21). A 1% suspension of E washed three times in neat FCS was added in equal volumes to a portion of the nylon-purified lymphocytes at a concentration of 5×10^6/ml. The mixture was incubated at 37°C, 15 min and then centrifuged at 400 *g* for 5 min. Thereafter the packed cells were kept on ice for 60 min. About half the supernatant was removed, then the cells were carefully resuspended and layered onto Ficoll-Isopaque solution (FI) (22) using an equivalent of $5\text{–}7.5 \times 10^6$ lymphoid cells/2 ml FI in a 15 ml conical centrifuge tube. The tubes were spun at 40C *g* for 30 min, the pelleted cells, E rosette forming, and the nonrosette forming, interphase cells, were collected separately and washed three times with TH plus 2.5% FCS. Each fraction, together with the same donor cells which had not been rosetted, but similarly prepared on FI, were then treated with Tris-ammonium chloride solution (23) to lyse sheep erythrocytes and remaining human erythrocytes. Each preparation was washed a further three times with TH plus 2.5% FCS. A portion of cells from each fraction and from the unfractionated cells from each donor was then recombined with sheep erythrocytes and the rosetting method repeated to check the numbers of E rosetting cells (T) in each preparation. The number of rosette-forming cells was estimated on wet and fixed preparations stained with May-Grünwald Giemsa (MGG) in parallel. At least 200 lymphoid cells were counted in each preparation, those with 4 or more attached sheep erythrocytes were counted as positive. All rosette-forming cells had the morphological appearance of small lymphocytes.

Complement Receptor.—The majority of nonthymus processed peripheral lymphoid cells possess a receptor for activated complement C3 (14, 20, 24, 25). Unfractionated cells were assayed for this receptor as described for human cells by Mellstedt et al. (26). Equal volumes (5 ml) of a 5% suspension of E washed three times and 19S anti-Forssman antibody (Statens Bakteriologiska Labotorium, Stockholm) in subagglutinating concentration were mixed and incubated at 37°C for 60 min with periodic shaking. The cells were centrifuged, washed two times with TH, and then diluted to 5 ml in TH. 2.5 ml were used for EA rosette formation (E coated with IgM antibody without complement). To the remaining sheep erythrocytes an equal volume of human AB serum diluted 1:20 in TH was added. After mixing, the cells were incubated a further 60 min at 37°C with occasional shaking. The cells were then washed two times in TH. This preparation (erythrocytes coated with IgM antibody plus complement) is termed EAC. EA and EAC were then diluted 10 times in TH. Lymphoid cells washed free of serum in TH, were used at a concentration of 1×10^6/0.25 ml and added in equal volumes to EA and EAC. The mixtures were centrifuged at 100 *g* for 6 min and then incubated at 37°C for 15 min. Half the supernatant was removed, the cells resuspended, and counted. Estimates were performed on 200 cells from each preparation and lymphoid cells with 4 or more attached E were considered rosette forming. Counts were performed on wet and fixed preparations stained with MGG.

Staining of Effector Cells.—Preparations from unfractionated and fractionated lymphocytes were checked for contaminating phagocytic cells after staining with acridine orange (27). Differential counts were made on all preparations stained with MGG.

Cell-Mediated Immunity.—Cell-mediated immunity was assayed in the microplate test as described previously (9, 16, 28).

Tissue Culture Medium.—Target cells were cultured in monolayers using Parker 199 medium containing 10% FCS, antibiotics (100 IU penicillin and 100 μg streptomycin/ml), and glutamine 0.3 mg/ml. For lymphoid cells medium 199 was diluted 1:1 with RPMI medium 1640 containing 20 mM Hepes buffer, and supplemented with FCS, antibiotics, and glutamine.

Target Cells.—Two cell lines formed the basic test material, T24 (9, 29), derived from TCC and HCV/29 derived from a nonmalignant specimen of bladder epithelium, (established by Dr. J. Føgh, Sloan Kettering Institute, New York). A primary culture of TCC, J82, was tested in passages 4–8. As a nonbladder target cell control a primary culture of metastatic melanoma MEL-1 was used in passages 13–16. All cultures were maintained free of mycoplasma.

Single cell suspensions were prepared by trypsinization with 0.02% EDTA + 0.05% trypsin and washed three times in medium. The cells were added to microplates (Falcon 3034) to give 30–40 cells/well. These were then incubated 3–4 h at 37°C in humidified air + 5% CO_2 during which time cell attachment was completed, plating efficiency of the target cells was $\geq$90%. The plates were then inverted for 20 min and the medium dumped off. Effector cell preparations in concentrations of 1 and 0.5 × 10^6 cells/ml were then added in 10 μl volumes to the target cells. 12 wells were used for each effector cell preparation at a given concentration.

The plates were incubated for 24 h at 37°C in humidified air + 5% CO_2. At termination of the experiment they were inverted for 30 min, the medium dumped off, and then washed gently with PBS, pH 7.2. The residual target cells were fixed and stained with MGG.

Estimation of Cytotoxicity.—In each experiment the arithmetic mean of the surviving target cells in wells which had contained patients' lymphoid cells was compared with that in wells having contained control donors' cells at equivalent concentrations, for each effector cell preparation. The significance of differences was estimated by Student's t test with $P \leq 0.05$ considered as significant. Cytotoxicity is expressed as the percent of reduction = $(1 - P/C) \times 100$ (P = mean surviving target cells after incubation with patients' lymphoid cells; C = mean surviving target cells after incubation with control donors' lymphoid cells).

Antibody-Dependent Cytotoxicity.—Unfractionated and fractionated lymphoid cell preparations from two patients and one control were tested for ability to mediate antibody dependent lysis of target cells (30). 1 × 10^6 Chang liver cells in 1-ml tissue culture medium were labeled with 100 μCi sodium chromate (specific radioactivity 100–300 mCi/mg Cr) for 40 min at 37°C. The cells were then washed three times in medium. Cytotoxicity was tested in 15-ml conical plastic centrifuge tubes each containing 25 × 10^3 labeled target cells. Lymphoid cells were tested at a ratio of 30:1 to the target cells. Heat-inactivated (56°C, 60 min) rabbit anti-Chang serum was used at a final diluation of 3 × 10^{-4}. The total incubation volume was 1.5 ml. Each parameter was tested in duplicate tubes. The tubes were incubated 18–20 h in humidified air + 5% CO_2. Cytotoxicity is expressed as the corrected isotope release after subtraction of the background release in target cell controls incubated with medium only (range 18–23%). Maximum variation between duplicate tubes was ±3%.

PHA-Induced Cytotoxicity.—Unfractionated and fractionated lymphocyte preparations were tested for cytotoxic activity towards Chang cells in the presence of phytohemagglutinin (PHA) (30). These tests were carried out in parallel with those for antibody-mediated cytotoxicity. PHA (purified PHA-W, Wellcome Research Labs., England) was added to the incubation mixtures in doses of 0.1 μg and 1 μg.

RESULTS

Characterization of Unfractionated and Fractionated Populations According to Surface Markers.—Purified lymphocyte preparations were characterized according to two surface markers, capacity to form E and EAC rosettes. As shown

in Table I these preparations from both patients and control donors had the proportions of cells carrying these markers described previously for normal human peripheral blood lymphocyte preparations (13, 14, 20, 24). After sedimentation of E rosette-forming cells in FI and removal of E by NH_4Cl lysis, the pelleted cells were found to contain 90% or more cells capable of reforming E rosettes. In this fraction $\leq 4\%$ of the cells formed EAC rosettes. Conversely, the interphase fraction was seen to contain $\leq 7\%$ E rosette-forming cells, but 76% of these cells could form EAC rosettes. These observations are summarized in Table I. In all eight experiments the average numbers of E rosette- and EAC rosette-forming cells in both unfractionated preparations and in the two fractions from patients and controls were similar. The mean values with the standard deviation were as follows: for E rosettes unfractionated cells 76 ± 6, pellet 92 ± 7, and interphase 9 ± 5; and for EAC rosettes unfractionated cells 25 ± 3, pellet 3 ± 1, and interphase 76 ± 2.5. Representative values for one patient and control are shown in Table I. 50–70% of the starting populations of E rosette- and non-E rosette-forming cells were recovered after fractionation. Morphologically the cells obtained after nylon wool purification and fractionation were $\geq 95\%$ lymphocytes.

TABLE I

Surface Markers of Lymphocyte Fractions

Donor	Fraction	Rosette-forming cells	
		EAC	E
		%	%
Normal	Unfractionated	18	74
	Pellet	4	90
	Interphase	76	7
Ca. bladder	Unfractionated	20	72
	Pellet	4	90
	Interphase	76	6

Cytotoxic Potential of the Cell Fractions.—It has previously been reported (16) that lymphocytes from both normal donors and patients prepared by FI cause a high nonspecific cytotoxicity in the microplate assay as compared with the same donors' cells prepared by nylon wool purification. This cytotoxicity does not relate to differences in purity of the effector cell preparations, and becomes manifest at high effector cell: target cell ratios and after incubation periods of 2 days or more. To avoid these nonspecific effects in this series of experiments the effector target cell ratios were kept relatively low (125:1 and 250:1) and the incubation time of the assay was also shortened to 24 h. Under these conditions the mean surviving number of target cells incubated in medium only compared well with that in wells having contained control effector cells (Table II).

TABLE II

Tumor-Directed Cytotoxicity of Different Lymphocyte Fractions

Target	Lymphocyte fraction	E:T	Surviving target cells per well*		Reduction‡	Compared with control
			Patient	Control		
			(*mean* ± *SE*)		%	*P*
J82	Unfractionated	250:1	46 ± 2.9	65 ± 2	29	<0.001
		125:1	60 ± 2.3	70 ± 2.7	14	0.01
	Pellet	250:1	63 ± 3	67 ± 3.5	6	N.S.§
		125:1	76 ± 3.3	73 ± 2.5	0	
	Interphase	250:1	21 ± 1.1	66 ± 2.5	68	<0.001
		125:1	34 ± 4	70 ± 3.5	51	<0.001
T24	Unfractionated	250:1	57 ± 2	75 ± 3.2	24	<0.001
		125:1	56 ± 2.2	71 ± 3.7	21	<0.01
	Pellet	250:1	64 ± 1.9	63 ± 2.3	0	
		125:1	63 ± 1.9	62 ± 1.3	0	
	Interphase	250:1	24 ± 1.9	57 ± 2.7	58	<0.001
		125:1	33 ± 3.5	70 ± 2.5	53	<0.001
HCV/29	Unfractionated	250:1	50 ± 4.5	54 ± 3.8	7	N.S.
		125:1	52 ± 3.3	53 ± 2.6	2	N.S.
	Pellet	250:1	54 ± 3	56 ± 3.8	4	N.S.
		125:1	61 ± 2.3	58 ± 2.8	0	
	Interphase	250:1	50 ± 3.7	52 ± 3.4	4	N.S.
		125:1	50 ± 4.6	54 ± 3	7	N.S.
MEL-1	Unfractionated	250:1	28 ± 2.4	28 ± 2	0	
		125:1	36 ± 6	32 ± 2.4	0	
	Pellet	250:1	44 ± 4	42 ± 3.6	0	
		125:1	40 ± 3.2	40 ± 2.4	0	
	Interphase	250:1	36 ± 3.2	24 ± 2.4	0	
		125:1	36 ± 2	32 ± 3.2	0	

* Surviving target cells incubated with medium only (mean ±SE) J82, 62 ± 4; T24, 61.5 ± 3; HCV/29, 56 ± 5; and MEL-1, 44 ± 3.

‡ Estimated as described in Materials and Methods.

§ N.S. not significant.

Before fractionation lymphocytes from six of the eight patients with TCC showed tumor-specific cytotoxicity (Table III). After fractionation, all measurable cytotoxicity was recovered in the interphase (non-E rosetting) preparation in all cases. In four patients this fraction gave increased levels of specific cytotoxicity as compared with the unfractionated cells at equivalent effector: target cell ratios. This is exemplified in Table II and was observed in three additional experiments. In two experiments the cytotoxicity produced by the interphase fraction remained at the same level as in the unfractionated cells. No tumor-specific cytotoxicity was detectable in the pelleted fraction (E

rosette-forming cells) of any patient. Lymphocytes from the control donors showed no specific activity either before or after fractionation.

Functional Activity of Cell Fractions in Antibody-Mediated and PHA-Induced Cytotoxicity.—As shown in Table IV cytotoxicity mediated by rabbit IgG antibody to Chang cells was obtained in this assay with the unfractionated and interphase cells only. The pelleted (E rosette-forming population) showed no significant reactivity. In fact a clear enhancement of cytotoxicity was produced by the interphase cells. The results are in agreement with those previously published (15) and show that the lymphoid cell type predominating in this reaction has non-T characteristics (4, 5).

TABLE III

Summary of Patients with TCC Tested in Fractionation Experiments

Patient	Clinical situation*	Tumor-specific cytotoxicity of lymphocyte fractions‡		
		Unfractionated	Interphase	Pellet
1	T3 M3	28	33	0
2	T2 M2	18	62	0
3	T1 M1	0	0	0
4	T3 M3	30	32	0
5	T2 M1	17	30	0
6	T4 M3	29	68	0
7	T4 with metastases	0	0	0
8	T2 M2	15	37	0

* For details see Materials and Methods. All patients had tumor present at the time of testing.

‡ Percent reduction at an effector:target cell ratio of 250:1.

It has previously been demonstrated that the E rosette-forming cells have an increased reactivity to PHA as measured by DNA-synthesis (13). The capacity of purified lymphocytes before and after fractionation to participate in PHA-induced cytotoxicity is shown in Table V. The pelleted cells (E rosette-forming fraction) show a moderately increased reactivity in this test as compared with the unfractionated cells. The interphase cells give a reduced but significant cytotoxic effect also.

DISCUSSION

These results confirm and complement previous observations (12) which showed that the effector cells in this tumor-specific reaction reside in the non-thymus-derived population of peripheral lymphocytes. Thus cytotoxicity was lost after removal of lymphocytes with Fc receptors and/or surface Ig on columns coated with human Ig and rabbit anti-human Ig in excess.

In the present series of experiments it is seen that removal of lymphocytes capable of forming direct E rosettes does not reduce existing cytotoxicity, in

TABLE IV

Activity of Lymphocyte Fractions in Antibody-Mediated Cytotoxicity

Fraction	Cytotoxicity*	
	Chang cells	Chang cells + AS‡
Unfractionated	7	26
Pellet	1	3
Interphase	10	40

* Isotope release corrected by subtracting release in lymphocyte-free controls. Incubation for 18 h.

‡ AS, antiserum present. See Materials and Methods.

TABLE V

Activity of Lymphocyte Fractions in PHA-Induced Cytotoxicity

Fraction	^{51}Cr release from Chang cells*		
	Medium	0.1 μg PHA	1 μg PHA
Unfractionated	1	20	29
Pellet	−2	26	38
Interphase	−1	14	8

* Corrected by subtracting release in lymphocyte-free controls. Incubation for 18 h.

fact increased reactivity is often found in the residual cells. The E rosette-forming cells in human blood have the characteristics of thymus-derived cells (13, 14, 20).

The lymphocyte populations were identified before and after fractionation according to a second surface marker, the capacity to bind EAC (14, 20, 24, 25). The distribution of this marker predominantly in the non-E rosette-forming interphase fraction provides additional evidence for the nonthymus origin of the effector cells in this tumor-specific reaction.

Morphologically non-T cells in human blood have been identified on the basis of surface Ig, the possession of receptors for activated complement, and for Ig aggregates (31). On a functional level cells carrying the C3 receptor are known to participate in antibody-mediated cell lysis (15, 32). In the latter type of reaction an intact Fc portion is required on the inducing antibody (33, 34) indicating that the effector cells carry a receptor for Fc. The loss of tumor-specific cytotoxicity after removal of lymphocytes with Fc receptors and/or surface Ig (12) indicate that the same cell type may be operative in this situation. Antibody-mediated cytotoxicity by nonimmune lymphocytes has been shown to require Fc receptor-bearing cell but not Ig-bearing cells per se (15, 35). A strict classification of the thymus-independent effector cells in this tumor-specific cytotoxicity must therefore await further functional analysis.

The increased levels of tumor-specific cytotoxicity often observed after the depletion of T cells speaks against, but does not formally rule out, T-cell partic-

ipation at the effector cell level. The pelleted E rosette-forming cells, although totally ineffective in tumor-specific cytotoxicity, retain their capacity to react in PHA-induced cytotoxicity. This suggests that there is no functional impairment of these cells due to preparative treatment. That the interphase cells (non-T) also respond, but to a lesser extent, in PHA-induced cytotoxicity, may be explained either by the capacity of B cells to respond to PHA (36) or on the basis of residual T cells in this fraction. That PHA can induce cytotoxicity in lymphocytes free of T cells has been described (37).

A sequential development of thymus-derived and thymus-independent effector cells has been described during the development of a viral induced murine tumor (2). The assay used was similar to that in the present experiments. In a ^{51}Cr release assay in a similar system (3) a predominantly T-cell cytotoxicity has been reported. The type of effector cell detected may therefore be influenced by the assay employed. However, the duration and intensity of antigenic stimulation in vivo during different phases of tumorigenesis may determine the type of effector cells which predominate. In the human patient with established TCC a prolonged contact with tumor antigen(s) can be inferred.

SUMMARY

Peripheral lymphocytes from patients with urinary bladder carcinoma and controls have been separated on the basis of rosette formation with sheep erythrocytes. The fractions were tested for tumor-specific cytotoxicity. The E rosette-forming cells of purity $\geq$ 90% respond well in PHA-induced cytotoxicity but are totally inactive in the tumor assay. The non-E rosette-forming cells (purity $\geq$ 91%) give enhanced activity in the tumor-specific cytotoxicity as well as in antibody-mediated target cell lysis in a model system. These data support the notion that the effector cells in cell-mediated immunity to carcinoma of the urinary bladder are members of the nonthymus-derived population of peripheral lymphocytes.

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