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Effector Cells in Natural Cytotoxicity Against Human Bladder Cancer Cell Lines*

Eda T. Bloom**, Marguerite Dunsworth-Browne and Diane Durkos-Smith

Department of Microbiology and Immunology, School of Medicine, University of California, Los Angeles, California, USA

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Summary. Human peripheral blood mononuclear cells obtained by ficoll-hypaque sedimentation were depleted of Fc-receptor-bearing (FcR+) cells. Cytotoxicity (direct killing of target cells by effector cells), tested in a 40 h assay, was significantly decreased against a variety of target cells. Tests in which no FcR+ cells could be detected were also positive for "natural killing" (NK) against a spectrum of target cells from normal donors. NK in this system was mediated by more than one subpopulation of lymphocytes. Monocytes probably did not play a significant role.

Decreasing the FcR+ cells in peripheral blood mononuclear cells in patients with bladder cancer and in controls did not reveal specific antitumour activity.

Key words: Natural cytotoxicity, Bladder cancer.

INTRODUCTION

Following the controversy surrounding the reports of "tumour-specific" cell-mediated immunity in cancer patients, and subsequent reports of its absence (19), much research focused on identification and characterisation of the cellular mediators of non-disease associated cytotoxicity. In humans the phenomenon, known as spontaneous (11) or natural (10) killing, was shown to be mediated by lymphocytes of the null cell subset which possessed Fc and C3 receptors, but no other surface markers. More recently, West et al. (21) have shown that FcR+ cells which also form low affinity E-rosettes may be the responsible cellular mediators. Depletion of FcR+ cells from peripheral blood lymphocytes decreased or eliminated the natural killing effect (20, 21). Though the original observations of natural killing (NK) activity were made in long-term assays against adherent target cells (19), the cellular dissections were done primarily in short-term assays against lymphoid, lymphoblastoid, or tumour derived target cells.

The present study was undertaken in an attempt to determine whether depletion of FcR+ cells would reveal bladder tumour associated cytotoxicity while decreasing or eliminating NK. NK in this report is used to refer to cytotoxicity which is not attributable to disease status or other obviously definable antigenic characteristic. Depletion of FcR+ cells did not eliminate such NK, and it was found that FcR negative lymphoid cells may also mediate natural killing or mature into active effector cells in the long term assay.

MATERIAL AND METHODS

Effector Cells (EC)

Heparinised venous blood was collected from normal donors, from patients with nonmalignant genitourinary disorders (2 incontinence procedures; 2 prothesis insertions, 2 benign prostatic hypertrophies, 1 renal obstruction, 1 benign epithelial atypia, 1 epididymitis, and 1 interstitial cystitis); bladder cancer patients (3 superficial, 4 invasive, and 5 metastatic), and from patients with other cancers (2 prostatic carcinomas, 1 renal cell, and 1 testicular embryonal cell). Mononuclear cells were obtained by sedimentation over ficoll-hypaque (FH) (4). Phagocytic cells were removed

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** Current address: GRECC 691/11G, V.A. Wadsworth Medical Center, Los Angeles, CA 90073, USA

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with a magnet following a 30 min incubation with carbonyl iron (Fe, 10 mg/ml in medium with 25% fetal calf serum) with rotation at 37°C. Fc receptor bearing cells were depleted and recovered using the monolayer immune complex (MIC) fractionation of Targan and Jondal (20), or the erythrocyte-anti erythrocyte monolayer (EA) technique of Kedar et al. (12). Both techniques rely on combination of the FcR+ cells with rabbit antibody (IgG, Fc portion) previously attached to antigen in 60 mm plastic petri dishes. In the MIC method the antigen is human immunoglobulin, and in the EA method the "antigen" is sheep erythrocytes. Cells adherent to the MIC monolayer were incubated for 2 h at 37° with 2 ml protein A (150 μg/ml, Pharmacia, Piscataway, NJ) to competitively inhibit the FcR binding to the monolayer, in order to recover the FcR+ cells (20). Cell counts were done using Unopettes and trypan blue dye exclusion. The following cell surface markers were determined on the cell preparations: Sheep erythrocyte (E)-rosettes for T cells (6), EA rosettes for FcR+ cells (9), and membrane immunofluorescence for surface membrane immunoglobulin (SMIg) (14). Smears were prepared with a Cytocentrifuge and stained with Camco Quik Stain for cellular enumeration. For experiments in which lymphocyte preparations were cultured for 1-3 days, the cells were suspended at 10^6/ml and incubated in Eagle's minimum essential medium with non essential amino acids, 1% fungizone, 50 μg/1 gentamycin, and 10% heat inactivated fetal calf serum (MEM).

Cytotoxicity Test

Cell suspensions were adjusted to 10^7/ml in MEM and plated on target cells in 10 μl volumes in Microtest I plates (Falcon Plastics, Oxnard, (CA). All tests were run in triplicate as described (2, 3). Tests were run for 2 days (40 h) except where indicated. To terminate the tests, plates were washed, fixed with methanol and stained with Geimsa's. Cytotoxicity was evaluated visually, and the endpoint determined as that number of effector cells resulting in 50% survival of target cells relative to media controls. Cytotoxic indices were assigned as follows: 0 = no detectable cytotoxicity, 1 = 50% endpoint at 10^5 EC, 2 = at 5×10^4, 3 = at 2.5×10^4, 4 = at 1.2×10^4, 5 = at 6×10^3, and 6 = endpoint at 3×10^3.

Target cells were plated at least 12 h prior to addition of EC at a concentration to allow about 100-200 cells to adhere for testing. They were obtained from the following cell lines: T24 (5), 253J (7), and J82 (15), derived from bladder transitional cell carcinomas; HT29M (1), derived from a colon carcinoma; and Fl (8), derived from normal amnion. Lines were maintained in either MEM with 10% FCS (T24, HT29M, and Fl) or RPMI 1640 with 15% FCS (253J, J82). All cell lines were negative for mycoplasma contamination by the DAPI fluorescence stain method of Russell et al. (17).

All statistical analyses were by the Student's t-test.

Table 1. MIC and EA monolayer depletion of FcR+ cells and reduction of cytotoxicity in 10 normal donors

EC markers and function	Effector cell preparation		
	Ficoll-Hypaque only	Post MIC	Post EA monolayer
Markers: (percent ± SE)			
SMIg	9.8 ± 1.0	9.6 ± 1.1	8.8 ± 0.6
EA	17.2 ± 1.6	3.6 ± 0.6[c]	5.1 ± 0.8[c]
E	67.3 ± 2.8	68.5 ± 1.7	69.9 ± 2.2
Cytotoxicity: (cytotoxic index ± SE)			
against:			
T24	4.50 ± 0.62	2.33 ± 0.95[a]	2.80 ± 0.94
253J	2.94 ± 0.78	1.55 ± 0.71	3.00 ± 1.02
J82	4.20 ± 0.48	3.95 ± 0.72	4.30 ± 0.68
FL	3.55 ± 0.50	1.50 ± 0.61[b]	2.00 ± 0.66[a]
HT29M	4.55 ± 0.51	3.10 ± 0.67	2.80 ± 0.53[a]

[a] $p < 0.05$ compared to FH only

[b] $p < 0.01$ compared to FH only

[c] $p < 0.001$ compared to FH only

Table 2. Effect on cytotoxicity of phagocytic cell depletion (12 donors)[a]

Part	EC Preparation	Differential (% ± SE) LC	MONO	PMN	% Recovery (± SE)	Surface markers (% ± SE) E	EA	SMIg	Cytotoxicity (Index ± SE) T24	253J	J82	FL	HT29M
A	FH + MIC	90.1 ± 1.3	4.9 ± 1.0	4.9 ± 1.2	44.2 ± 2.6	66.1 ± 2.6	5.8 ± 0.6	8.2 ± 1.0	1.75 ± 0.44	0.72 ± 0.28	1.62 ± 0.45	0.86 ± 0.24	0.75 ± 0.13
	FH + Fe + MIC	97.1[d] ± 0.6	2.3[b] ± 0.8	0.2[d] ± 0.1	42.8 ± 1.8	62.9 ± 2.9	3.5 ± 0.8	9.0 ± 1.4	1.33 ± 0.33	1.04 ± 0.23	1.78 ± 0.42	1.40 ± 0.27	1.12[b] ± 0.16
B	FH	70.8 ± 2.0	11.8 ± 1.4	17.2 ± 3.0	100	63.3 ± 3.2	18.9 ± 1.9	7.9 ± 1.4	2.66 ± 0.33	1.45 ± 0.40	2.50 ± 0.84	2.68 ± 0.26	2.45 ± 0.19
	FH + Fe	92.9[d] ± 1.4	5.0[d] ± 1.2	2.8[d] ± 0.9	66.6 ± 2.1	65.2 ± 2.8	18.2 ± 2.2	8.4 ± 1.4	3.16 ± 0.27	2.00 ± 0.36	3.57 ± 0.22	3.50[c] ± 0.26	2.95[b] ± 0.18

[a] Phagocytic cells were depleted by incubation with carbonyl Fe, and removal with a magnet
[b] $p < 0.05$ compared to corresponding group without Fe
[c] $p < 0.025$ compared to corresponding group without Fe
[d] $p < 0.005$ compared to corresponding group without Fe

RESULTS

Reduction of Cytotoxicity by Depletion of FcR+ Cells

Cytotoxicity by EC following MIC or EA monolayer depletion of FcR+ cells was compared in 10 normal donors. Both depletion methods resulted in decreased cytotoxicity. Although neither method eliminated cytotoxicity, the MIC technique was more efficient for the depletion of FcR+ cells and the reduction of cytotoxicity (Table 1). The MIC technique was therefore used in subsequent experiments.

Monocyte Depletion

In order to determine if monocyte depletion by MIC fractionation was responsible for the decreased cytotoxicity, phagocytic cells were

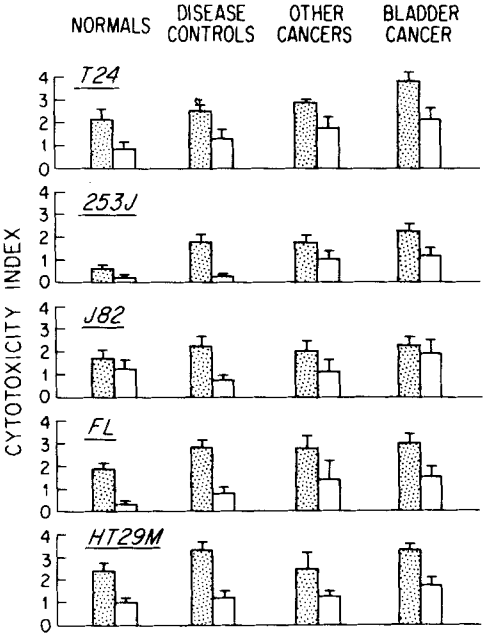

Fig. 1. Histogram bars show cytotoxic indices against 5 target cells by lymphocytes obtained by FH sedimentation pre (▨) and post (□) MIC depletion of FcR+ cells. Lines indicate standard errors. P values comparing cytotoxicity by native and FcR+ cell depleted populations by the Student's t-test for normal individuals (n = 13) were: T24, $p < 0.025$; 253J, $p < 0.005$; J82, $p < 0.20$; Fl, $p < 0.005$; and HT29M, $p < 0.005$; for disease controls (n = 10): T24, $p < 0.01$; 253J, $p < 0.001$; J82, $p < 0.005$; Fl, $p < 0.005$; and HT29M, $p < 0.005$; for patients with other cancers (n = 4): T24, $p < 0.10$; 253J, $p < 0.10$; J82, $p < 0.20$; Fl, $p < 0.20$; and HT29M, $p < 0.10$; and for patients with bladder cancer (n = 12): T24, $p < 0.025$; 253J, $p < 0.025$; J82, $p < 0.40$; Fl, $p < 0.005$; and HT29M, $p < 0.005$

reduced by incubation with carbonyl iron (Table 2). Though decreasing the monocytes did not greatly affect the MIC fractionated cells (part A), it increased the cytotoxicity in the FH separated cells (part B). However, conversion of the differences in cytotoxic indices to EC numbers indicates that the difference can be accounted for by the percentage recovery of cells following Fe treatments, and resulting enrichment of cytotoxic cells. Fe treatment was used in subsequent experiments.

Depletion of FcR+ Cells in Bladder Cancer Patients Compared to Other Groups

Table 3 and Fig. 1 show that depletion of FcR+ cells decreased cytotoxicity in patients with nonmalignant genitourinary diseases, bladder cancer, and other cancers, as well as normals. However, no obvious differences emerged after depletion to indicate bladder cancer specific cytotoxicity. In some cases (e. g. T24), a difference between donor groups prior to MIC fractionation was obscured in FcR+ depleted EC. Although differences in numbers of FcR+ cells between donor groups occurred before and after depletion, these did not correlate with, nor could it explain the resultant cytotoxicity by the different groups against the different cell lines.

Kinetics of Cytotoxicity by FcR+ Cell Depleted EC

Since the cytotoxicity test was run for 2 days, it was possible that cellular receptors were being generated by differentiation during the test. To approach this question, FcR+ depleted cell populations were cultured 1, 2, or 3 days before marker enumeration and plating on the target cells for 2 additional days. Simultaneously, in parallel assays, the same cell preparations were incubated with the target cells for 1, 2, 3, 4 or 5 days on the cytotoxicity plates. (Wells were fed with 10 µl of media as required). Table 4 and Fig. 2 show the results of these experiments, with EC from four normal individuals. As anticipated, increasing the time of incubation with target cells increased the cytotoxicity (Fig. 2). Moreover, culturing the EC alone for 1-3 days prior to plating on target cells also increased the cytotoxicity against 253J and Fl, but not significantly against HT29M. None of the cell preparations used in these test had recovered or generated Fc receptors during incubation (Table 4).

Recovery of MIC-Adherent Cells

Cells adherent to MIC plates were recovered by incubation of the plates with protein A. Preliminary attempts to recover MIC-adherent cells yielded fewer lymphocytes positive for any of the surface markers (Table 5). This could have resulted from the protein-A recovery procedure. Nevertheless, the cells recovered when readjusted to 10^7/ml, were as cytotoxic as the unfractionated cells, and significantly greater than the nonadherent fraction recovered from the MIC plates. However, the killing was not as strong as would be expected if cytotoxic cells were greatly enriched.

DISCUSSION

Natural killing (NK) was originally observed in long term cytotoxicity assays in attempts to detect human tumour associated reactivity (19). Since natural cytotoxicity as studied using the 4 h chromium release assay may or may not be applicable to possible detection of cancer related reactivity in patients with solid tumours, NK was studied using the long term cytotoxicity assay. The term "natural killing" in this report is applied to cytotoxicity which cannot be attributed to disease status or explained using any obviously definable clinical or antigenic characteristic.

Depletion of peripheral blood mononuclear cells of Fc-receptor bearing (FcR+) cells consistently had no detectable effect on E rosetting cells and surface membrane immunoglobulin positive cells (SMIg+), but was always correlated with decreased cytotoxicity against a variety of adherent, cultured target cells in a 40 h assay (Table 1). Reduction of FcR+ cells and cytotoxicity was similar with blood from normal donors and patients with nonmalignant genitourinary disorders, bladder cancer, and other cancers. No bladder cancer specific cytotoxicity against transitional cell carcinoma derived cell lines emerged (Table 3, Fig. 1). In the results presented in Table 3, both bladder cancer patients and patients with nonmalignant genitourinary diseases had greter numbers of EA+ cells than the other groups both before and after FcR+ cell depletion. This was not necessarily correlated with greater killing against the different target cells.

In confirmation of previous work (3), monocytes did not play a significant role in the natural killing phenomenon. The lymphocytes mediating the NK effect, however, appear to be derived from more than one subpopulation. FcR+ cells either mediate NK, or fulfilled some other contributing function (regulatory?). Since E rosetting cells did not increase with FcR+ cell depletion and were recovered from MIC plates (Table 5), some of the FcR+ cells were probably FcR+ T cells as also suggested by West et al. (21). Such T cells probably bore Fc receptors for IgG since the rabbit antiserum used for

Table 3. Surface markers on EC obtained from patient groups and normal donors[a] (percent + SE)

Group	No.	Recovery from MIC	Pre MIC			Post MIC		
			SMIg	EA	E	SMIg	EA	E
Normals	13	46.3 ± 3.1	11.4 ± 1.6	12.4 ± 1.3	64.1 ± 3.0	11.2 ± 1.5	3.5 ± 0.6	64.5 ± 2.6
GU disease controls	10	35.9 ± 3.1	9.2 ± 1.4	20.3 ± 3.3	58.8 ± 3.9	10.6 ± 1.1	5.7 ± 1.4	60.3 ± 3.7
Other cancers	4	40.0 ± 3.2	8.5 ± 1.6	12.5 ± 4.1	71.8 ± 3.9	6.5 ± 0.9	2.2 ± 0.8	69.0 ± 4.8
Bladder cancer	12	38.1 ± 4.4	7.1 ± 1.7	21.3 ± 2.7	64.8 ± 4.8	7.4 ± 1.7	6.1 ± 1.2	64.6 ± 5.3

[a]Cytotoxicity by these groups of EC is shown in Chart 1

Table 4. Cell populations and surface markers of mononuclear cells used in kinetics and pre-culture experiments (percent)[a]

Donor	Characteristics	Effector cells				
		Pre MIC	Post MIC, cultured (days)			
			0	1	2	3
A	Lymphocytes	88	96	QNS[b]	99	100
	Monocytes	12	4	QNS	1	0
	E	71	60	72	76	80
	EA	19	1	0[c]	ND[d]	0
	SMIg	7	7	7	14	7
B	Lymphocytes	88	99	100	98	94
	Monocytes	12	1	0.5	2	6
	E	59	67	67	88	79
	EA	14	2	2	ND	0
	SMIg	10	11	8	12	7
C	Lymphocytes	96	99	100	99	100
	Monocytes	4	3	0	0	0
	E	66	72	70	70	59
	EA	12	3	0	0	0
	SMIg	8	8	9	2	5
D	Lymphocytes	94	96	97	QNS	99
	Monocytes	6	4	3	QNS	0
	E	59	55	50	71	64
	EA	21	3	1	0	1
	SMIg	8	5	6	7	6

[a]Effector cells were cultured in vitro in MEM for 0-3 days prior to use in cytotoxicity tests. Results of the cytotoxicity tests are shown in Chart 2

[b]Quantity not sufficient

[c]No cells of indicated type were detectable in 200-600 counted

[d]No data

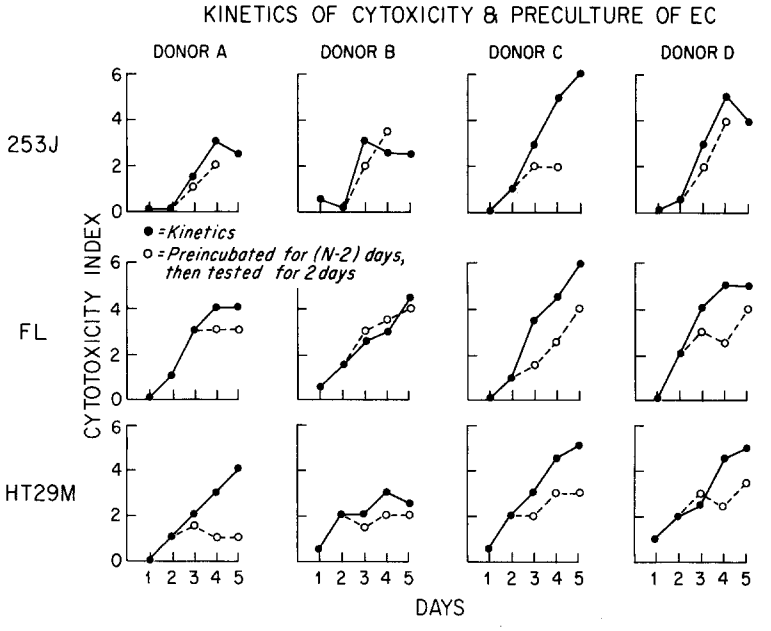

Fig. 2. Cytotoxic indices against each of 3 target cells are shown with 4 donors as a function of time of incubation with targets (o——o), and following preculturing for (n-2) days and incubation in the cytotoxic test with targets for 2 more days (o---o). (The total number of days including preculture and cytotoxicity test is shown on the abscissa.) Effector cell populations are described in Table 2

Table 5. Cytotoxicity by cells which had been adherent to MIC plates (7 donors; percent ± SE)

Cell population	Differential		% Recovery	Surface markers			Cytotoxicity		
	LC	MONO		E	EA	SMIg	253J	FL	HT29M
Pre MIC	92.8 ± 2.3	6.7 ± 2.2		69.3 ± 4.0	17.2 ± 3.4	9.3 ± 1.6	1.57[d] ± 0.48	3.64[d] ± 0.47	2.85[d] ± 0.55
MIC depleted	97.7 ± 0.7	2.1 ± 0.6	49.8 ± 3.3[b]	71.1 ± 4.2	4.5 ± 1.0	9.7 ± 2.1	0.92[e] ± 0.20	1.28[e] ± 0.21	1.07[e] ± 0.07
MIC recovered	95.6 ± 1.71	4.4 ± 1.71	15.1 ± 3.6[c]	31.6 ± 8.3	5.4 ± 2.3	4.0 ± 1.2	2.42[f] ± 0.27	3.42[f] ± 0.31	2.42[f] ± 0.38

[a] Cells which had been adherent to MIC plates were recovered by incubating the plates with protein A

[b] Percent recovery of pre-MIC cells

[c] Percent recovery of cells adherent to MIC plates

[d] Cytotoxicity pre-MIC: different from e (MIC-depleted), $p < 0.005$, except on 253J, with $p < 0.20$

[e] Cytotoxicity with MIC-depleted cells: different from f (MIC-recovered), $p < 0.005$

[f] Cytotoxicity with MIC-recovered cells: not statistically different from d (pre-MIC) ($p < 0.10$, $p < 0.40$, $p < 0.30$ on 253J, FL, and HT29M respectively)

depletion was hyperimmune and mostly IgG. However, NK can occur with no FcR+ cells present in the EC preparation (Table 4, Fig. 2). FcR-negative T and/or B cells are thus almost certainly also involved. Natural killing against adherent human target cells in a 40 h assay was not the result of a single subpopulation of lymphocytes, but could be mediated separately by more than one subgroup or via cellular interaction. The latter possibility was indicated by the fact that the MIC-adherent cells were cytotoxic, but not enough to account for all the cytotoxicity in the unfractionated cells (Table 5). This was also suggested by Pape et al. (16).

In the kinetics study, FcR+ cell depleted EC showed cytotoxicity not only with longer incubation with the target cells, but also when precultured for 1 to 3 days in medium alone (frequently with no FcR+ cells detectable). This latter increase occurred with target cells 253J and Fl, but not with HT29M (dashed lines, Fig. 2) in all four normal donors tested. If the EC were stimulated by the foetal calf serum during preculture period, then the resulting very strong primary response

showed selective increased cytotoxicity against 2 of the 3 targets, implying differing concentrations of foetal calf serum antigens on the target cells. However, the observed increased cytotoxicity may have resulted from activated NK activity in the FcR+ depleted EC population, with specificity as suggested by Takasugi et al. (18). In either case, the observed NK phenomenon in vitro is the result of more than one lymphocyte population, when measured in the long-term cytotoxicity assay against cultured human cells. FcR+ are probably involved, but not solely responsible for NK. Recently, Koide and Takasugi (13) have suggested that this is the case in short term assays as well.

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Dr. E. T. Bloom
GRECC 691/11G
V. A. Wadsworth Medical Center
Los Angeles, California 90073
USA