IN VITRO INDUCTION OF CYTOTOXIC EFFECTOR CELLS WITH SPONTANEOUS KILLER CELL SPECIFICITY

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Recent cytotoxicity studies in humans have shown that normal individuals have a defined lymphocyte subpopulation which is spontaneously cytotoxic to in vitro propagated cell lines (1-4). This phenomenon we have earlier called spontaneous lymphocyte-mediated cytotoxicity and the involved effector cell spontaneous killer (SK)[1] cell (1). In the mouse a similar phenomenon has been designated natural cytotoxicity (5). The human SK cell has been shown to express receptors for IgG (Fc receptors) and partly receptors for split products of the third complement factor (C3 receptors), whereas they do not express surface bound immunoglobulin (1, 4, 6). Whether the SK cells express receptors for sheep erythrocytes (RBC), a human T-lymphocyte marker, is a more controversial question. In most reports most SK activity is to be found in the non-sheep RBC binding cell fraction (1-3) whereas West et al. have demonstrated that SK cells can be made to bind sheep RBC under certain optimum conditions (4). Thus, from available information one can conclude that SK cells have Fc receptors and that they may or may not express sheep RBC receptors and that they are, so far, indistinguishable, by various fractionation procedures, from effector cells active in antibody dependent cellular cytotoxicity (7).

The biological significance of spontaneous cytotoxicity is, at present, unclear. According to the bias of the investigator, it may be conceived as anything ranging from an in vitro artifact to an important system in the regulation of the immune response or in the protection against tumor development (3). Beside the actual in vivo role SK cells may play, it is also unclear how they are generated, what type of target cell antigens they recognize, and by what mechanism they kill the target cells. In the present paper we have approached the questions of SK cell recognition and SK cell activation.

With regard to target cell recognition, we as well as others have found that SK cells from different individuals seem to have a similar target cell specificity as they consistently kill some cell lines better than others. Recently we have been able to extend these studies to show that SK cells preferentially kill cell lines that are derived from leukemic tumor cells as compared to cell lines derived from normal lymphocytes (8 and Table II). In the present work we have taken advantage of this relative target cell specificity to demonstrate that killer cells with a similar specificity may actually be generated in vitro. We have thus found that stimulation of peripheral lymphocytes with autologous or allogeneic B-cell lines for a 5-day period leads to a strong induction of

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[1] *Abbreviations used in this paper*: ADCC, Con A, concanavalin A; LDCC, lectin dependent cellular cytotoxicity; MLC, mixed lymphocyte culture; PBS, phosphate-buffered saline; RBC, erythrocytes; RSI, relative specificity index; SK, spontaneous killer; SRBC, sheep erythrocytes.

cytotoxic cells and that these effector cells kill the same target cells as those which are susceptible to SK cell cytotoxicity. We find this interesting as it may reflect how SK cells are generated in vivo as autologous lines induce cytotoxicity as well as allogeneic lines. If so, that would further define the nature of the SK system as several earlier reports have dealt with the mechanism and characteristics of B-cell line stimulation (9–13).

Materials and Methods

Purification of Lymphocytes. Lymphocytes were purified by centrifugation of heparinized whole blood on Ficoll-Hypaque gradients (14). Phagocytic cells were removed by the iron carbonyl powder technique (14).

Maintenance of Cell Lines. Cell lines were maintained as suspension cultures in RPMI-1640 medium supplemented with antibiotics and 10% fetal calf serum. Cell cultures were fed twice weekly.

Stimulation with B-Cell Lines. Stimulation cultures were set up in upright Corning 25 cm^2 tissue culture flasks (no. 25100). Responding lymphocytes were at a concentration of 1×10^6/ml with a responder:stimulator ratio of 2:1 or 3:1. Cultures were kept for 5 days at tissue culture conditions, whereafter the number of viable cells was calculated by trypan blue exclusion and the amount of DNA synthesis assayed by [^{3}H]thymidine incorporation in 0.1 ml of cultures (see below). In case of poor viability on the part of the responding cells, these were run on Ficoll-Hypaque gradients to remove dead cells and cell debris. The stimulating B-cell lines were established from normal individuals by Doctors C. Spina and M. Jobin at the Department of Microbiology and Immunology, University of California at Los Angeles, and were mitomycin C treated at a concentration of 50 μg/ml for 20–30 min at 37°C and washed three times before used. Cultures were set up in RPMI-1640 medium with 20% heat-inactivated autologous serum. Unstimulated control lymphocytes were tissue cultured for the 5-day period at a higher cell concentration of $2\text{–}4 \times 10^6$/ml as those cells otherwise would show poor viability on day 5.

Cytotoxicity Tests. Cytotoxicity tests were performed by ^{51}Cr release in standard V-shaped microplates (Cooke Laboratory Products Div., Dynatch Laboratories, Inc., Alexandria, Va., no. 1-220-25A) in a total vol of 150 μl RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum. Target cells were labeled with ^{51}Cr (New England Nuclear, Boston, Mass.) at a concentration of 200 μCi/5×10^6 target cells for 45 min at 37°C and washed three times. 10,000 target cells were used for each microplate well from which 50 μl supernate was harvested after an incubation period of 3-5 h and cytotoxicity estimated by the amount of released radioactivity. Cytotoxicity was calculated according to the standard formula:

$$\frac{\text{test release} - \text{spontaneous release}}{80\%\text{ of total label} - \text{spontaneous release}} \times 100.$$

Lectin dependent cellular cytotoxicity was also performed in V-shaped microplates as described for spontaneous cytotoxicity above. Concanavalin A (Pharmacia, Uppsala, Sweden) was used at a final concentration of 2 μg/ml which earlier had been found optimal in inducing DNA synthesis as well as lectin dependent cellular cytotoxicity (LDCC).

Calculation of Relative Specificity Index (RSI). To establish the specificity of various stimulated and unstimulated lymphocyte populations, we established an RSI. This index is based on the finding that certain cell lines are more susceptible to spontaneous cytotoxicity than others (Table I). We have picked Molt-4 as representative for susceptible target cell and Raji as representative for resistant target cells. Raji is thus an exception to the rule that tumor-derived targets in general are highly susceptible to SK cell cytotoxicity as can be seen in Table I. In calculating the index as described in Fig. 1, we establish an aggressor:target ratio dependent cytotoxicity curve for the Molt-4 and determine the amount of cytotoxicity at the early slope of this curve and the Raji cytotoxicity at the same aggressor to target ratio. At this point we estimate Molt-4 cytotoxicity and Raji cytotoxicity in a situation where there is an excess of aggressor cells. RSI is then calculated as Molt-4 cytotoxicity minus Raji cytotoxicity divided by Molt-4 cytotoxicity. In case Raji killing is higher than Molt-4 killing, this is indicated by a minus sign before the index. With equal killing of Molt-4 and Raji, the index will thus be 0.0 with only Molt-4 killing 1.0 and with 50% Raji killing, as compared to Molt-4 killing, 0.5. In calculation of RSI, no Molt-4 cytotoxicity values below 30% are accepted as these seldom represent slope values.

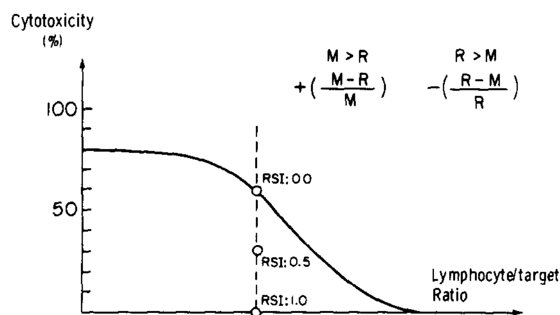

FIG. 1. For explanation of the relative specificity index, see Materials and Methods.

Assay for DNA Synthesis by Uptake of [³H]Thymidine. Of the stimulated cultures, 0.1 ml was harvested in triplicates in standard microplates and 2 μCi [^{3}H]thymidine put into each well. After an incubation period of 3-4 h, the microplates were frozen down to be eventually harvested in an automated multiple sample harvester and incorporated radioactivity measured by counting each sample in 5 ml of Econofluor (New England Nuclear).

Fractionation of Sheep RBC Binding Lymphocytes. Purified lymphocytes were mixed with sheep RBC at a lymphocyte: sheep erythrocyte (SRBC) ratio of 50:1 in 20% fetal calf serum. This cell mixture was then spun down in 50 ml round-bottomed centrifuge tubes and incubated for 15 min at 37°C and subsequently for 1 h at 4°C. Thereafter, the cells were gently resuspended and spun at 4°C on cold Ficoll-Hypaque. By repeating this procedure twice, non-sheep RBC binding lymphocytes, which were harvested from the interface, contained less than 5% of contaminating rosette-forming cells.

Fractionation of Fc Receptor-Bearing Lymphocytes. Fc receptor-bearing lymphocytes were fractionated with a monolayer immune complex fractionation technique as described elsewhere (15). Briefly, human IgG was dissolved in phosphate-buffered saline (PBS)-A (without divalent cations) to a final concentration of 0.5 mg/ml. 3 ml of this solution was poured into 60 × 15 mm tissue culture dishes (BioQuest, BBL, & Falcon Products, Becton, Dickinson & Co., Cockeysville, Md. no. 3002) and incubated therein for 30 min at room temperature and for 30 min at 4°C. Thereafter, the dish was washed three times with 2 ml of PBS-A and subsequently filled with 2 ml of a rabbit anti-human IgG serum. The antiserum was used at a final concentration of 1:20 and the dish was incubated for 30 min at room temperature for an additional 30 min at 4°C and then washed three times with PBS-A. Thereafter, purified lymphocytes, suspended in PBS-A with 10% fetal calf serum at a concentration of 5-7.5 × 10^6/ml, were added in a total vol of 2 ml to the plates which were then spun at 500 rpm for 5 min, turned 180°C, and spun for another 5 min. After the last centrifugation, the Fc receptor-bearing cells adhered to the plastic surface, and unbound cells could be collected by three washes with 2 ml PBS-A. Fc receptor positive cells could be partially collected accordingly: 2 ml of a protein A solution (Pharmacia, Uppsala, Sweden) at a concentration of 30 μg/ml was poured into each dish which then was incubated with slow rocking at 37°C for 2 h. During this time period, protein A competed with Fc receptors for binding sites on the IgG molecule, and part of the cell detached from the immune complex monolayers. The recovery of Fc receptor negative cells was approximately 71%, and of the Fc receptor positive cells approximately 14% of the initial cell number. Of plate-bound cells, approximately 36% could be eluted with protein A.

Results

In Vitro Parameters Influencing SK Cytotoxicity. It is well-known that many factors in the in vitro environment may influence SK cytotoxicity. It has thus already been shown that ammonium chloride, when used for RBC lysis, may decrease cytotoxicity as may preincubation of effector cells at 4°C or freezing of effector cells (16). Furthermore, we have found that autologous or heterologous RBC may increase cytotoxicity (M. Jondal, unpublished observa-

TABLE I

Effect of Autologous Serum on Spontaneous Cytotoxicity Mediated by Unfractionated Lymphocytes Against Cell Line K-562

Time of serum preincubation	Presence of serum in test medium	Trypsin treatment of lymphocytes	Cytotoxicity with serum dilutions								
			1	2	3	4	5	6	7	8	Control
h											
0	Yes	No	59	39	41	38	32	36	35	32	36
0	Yes	Yes	29	16	17	21	20	20	19	18	19
24	Yes	No	41	52	69	65	61	59	58	52	59
24	Yes	Yes	21	31	33	39	51	32	35	30	33
24	No	No	31	46	58	61	57	53	50	51	51
24	No	Yes	19	21	27	35	59	48	31	31	30

Lymphocytes were trypsinized for 20 min at 37°C with an enzyme concentration of 10 mg/ml. Untrypsinized or trypsinized lymphocytes were then tested for spontaneous cytotoxicity with or without preincubation in different serum concentrations starting from a final concentration of 1:3 with eight consecutive 1:3 dilution steps. Control test medium consisted of RPMI-1640 with 10% fetal calf serum.

tion) and that the serum source used in a 5-day culture may either increase or decrease SK cytotoxicity. We have made the following observations about the serum source: fetal calf serum can sometimes act as a mitogen and can thereby increase cytotoxicity, pooled human AB+ serum is inhibitory and little SK killing can be detected after 5 days, autologous serum can either be inhibitory or have no influence in the system. Table I exemplifies the effect of autologous serum on the SK cytotoxicity against the cell line K-562. One can see a slight increase of killing with high serum concentrations at time 0 whereas the same serum concentration is inhibitory at 24 h, but an increase of cytotoxicity can be noted at higher serum dilutions at this time point. This serum effect does not require serum present during the actual cytotoxicity test and is equally detectable on trypsinized lymphocytes. In our further tissue culture work involving culture stimulated lymphocytes and control lymphocytes, we chose to work with 20% autologous serum but it should be remembered that this serum concentration often decreases SK cytotoxicity of unstimulated control lymphocytes. In Table III this effect can be seen, with decrease of control cytotoxicity, with donors 1, 6, and 7 whereas donor 8 remains virtually unchanged.

Target Cell Specificity in the SK System. Table II summarizes the susceptibility of various target cells to spontaneous cytotoxicity as reported in more detail elsewhere (8). It can thus be seen that all of the tumor-derived target cells except Raji were classified as susceptible whereas all of the target cells derived from normal lymphocytes were classified as resistant. We defined resistance as equal to or less than 12% mean specific release in several repeat experiments during a 3–5 h ^{51}Cr release assay at a lymphocyte target ratio not higher than 20:1. The specificity as presently defined is thus of a relative nature as by altering the technical conditions such as by increasing the testing time or by increasing the number of effector cells some of the resistant lines will give a higher specific release than 12%. To further work with this relative specificity

TABLE II

Cell Lines Derived from Normal Lymphocytes are Less Susceptible to Spontaneous Cytotoxicity Than Most Cell Lines Derived from Leukemic Tumor Cells

Origin of cell line	Name of cell line	Number of cell lines tested	Susceptible/ resistant to spontaneous cytotoxicity
Burkitt's lymphoma (17)	P3HR-1	1	1/1
Burkitt's lymphoma (18)	BJAB	1	1/1
Burkitt's lymphoma (19)	Daudi	1	1/1
Burkitt's lymphoma (20)	Raji	1	0/1
Acute lymphocytic leukemia (21)	Molt-4	1	1/1
Acute lymphocytic leukemia (22)	HSB-II	1	1/1
Chronic myeloid leukemia (23)	K-562	1	1/1
Normal lymphocytes	See legend	15	0/15

Cell lines were tested for susceptibility to spontaneous cytotoxicity in a short-term (3-5 h) ^{51}Cr release assay. Susceptibility was defined in the present context as a higher specific mean release than 12%, in several repeat experiments, at a lymphocyte:target ratio not higher than 20:1. Cell lines derived from normal lymphocytes were either spontaneously transformed from Epstein-Barr virus seropositive individuals or transformed by the in vitro addition of virus by Doctors Celsa Spina and M. Jobin at the Department of Microbiology and Immunology, University of California at Los Angeles, and by Dr. Richard Gatti at the Department of Pediatric Oncology, Cedars-Sinai Medical Center, Los Angeles.

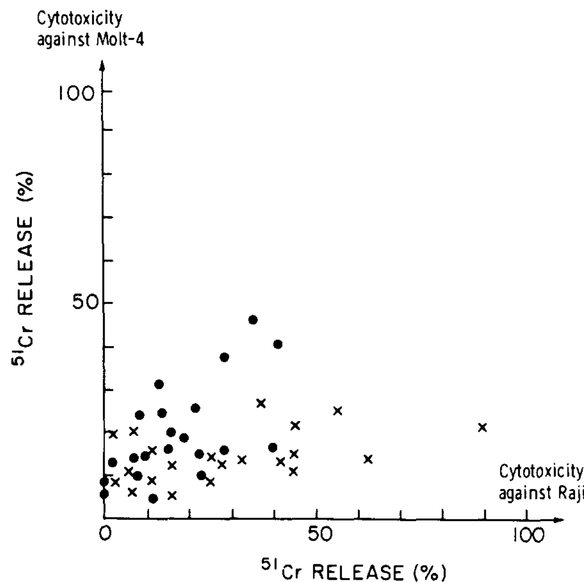

FIG. 2. Unfractionated lymphocytes or lymphocytes depleted for Fc and complement receptor positive cells by the EAC(7S) rosette sedimentation procedure (24) were tested for specific Con A dependent cellular cytotoxicity as described in Materials and Methods. ×, unfractionated, ●; Fc receptor negative.

of the SK system, we selected one line as representative for the susceptible group (Molt-4) and one line as representative for the resistant group (Raji) and defined an RSI as described in the Materials and Methods section. In Fig. 3 it can be seen that there is a tendency towards lesser specificity, i.e., more Raji

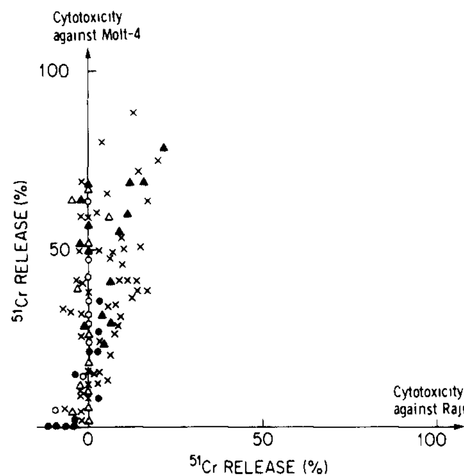

FIG. 3. Different unstimulated lymphocyte subpopulations fractionated as described in Materials and Methods were tested for spontaneous cytotoxicity against Molt-4 and Raji. ×, unfractionated; △, E receptor positive; ○, E receptor negative; ▲, Fc receptor positive; ●, Fc receptor negative (20 experiments).

killing with increasing Molt-4 cytotoxicity and that this seems to occur with all tested lymphocyte subpopulations. In later experiments with SRBC fractionated cells, we have seen that this also is true for non-SRBC binding lymphocytes although these cells appear as comparatively more Molt-4 specific in Fig. 3.

In Table VII we have calculated the RSI from 12 experiments with unfractionated lymphocytes and found a mean RSI of 0.84.

To exclude that the relative resistance on part of the Raji cell line was due to a general resistance to cell-mediated lysis, we tested the susceptibility of Raji cells and Molt-4 cells to concanavalin-A (Con-A) dependent cytotoxicity. From Fig. 2 in which we have plotted specific Con-A dependent Raji killing against specific Con-A dependent Molt-4 killing, it is clear that Raji cells are as susceptible to lysis as Molt-4 cells. With unfractionated cells there is relatively less Con A dependent cytotoxicity against Molt-4 as compared to killing with Fc receptor depleted cells which is related to the fact that there is a high SK background with the Molt-4 cell line. The results in Fig. 2 are furthermore in line with our earlier results showing that SK resistant target cells can be killed by LDCC and that relatively little LDCC can be detected against target cells that are very susceptible to spontaneous cytotoxicity and especially so with isolated Fc receptor positive lymphocytes (25).

In summary, these results with unstimulated "true" SK cells show that these cells do have a relative specificity which may be tumor related and that the specificity most probably is related to lack of recognition at the effector cell level as the resistant target cells become susceptible in the presence of Con A and finally that the RSI of SK cells when defined by killing against Molt-4 versus killing against Raji is 0.84.

Induction of Cytotoxicity and DNA Synthesis by Stimulation with B-Cell Lines. Stimulation of peripheral lymphocytes with mitomycin C-treated allogeneic or autologous B-cell lines result in increased DNA synthesis and induction of cytotoxic effector cells. In Table III a representative experiment is

TABLE III

Cytotoxicity against MOLT-4 by Spontaneous Killer Cells and by Killer Cells Induced by Stimulation of Unfractionated Lymphocytes with an Allogeneic B-Cell Line

Exp.	Donor	Unstimulated					Stimulated			
		Day 0		Day 5			Day 5			DNA Synthesis
		20:1	10:1	20:1	10:1	5:1	20:1	10:1	5:1	
		^{51}Cr release								*cpm/0.1 ml culture*
39	Macintosh	20	12	2	1	0	72	74	64	55,082 (194)
	Myers	51	41	ND			75	56	49	39,166 (ND)
	Roman	20	13	ND			68	40	31	66,820 (ND)
	Hopkins	37	26	ND			82	75	80	93,251 (ND)
	Luthardt	63	47	ND			87	83	89	82,587 (ND)
	Kechejian	35	22	22	9	5	81	78	79	66,136 (1,339)
	Flink	31	29	14	3	2	82	81	76	96,065 (658)
	Jondal	46	41	61	46	28	83	90	79	90,845 (2,964)

Lymphocytes were tested for spontaneous cytotoxicity against Molt-4 at days 0 and 5 and after stimulation for 5 days with an allogeneic B-cell line. Cell cultures were set up as described in Materials and Methods and DNA synthesis estimated from 0.1 ml of total cell culture, control values without stimulation are given in parentheses.

TABLE IV

Generation of Killer Cells with SK Specificity during Stimulation of Unfractionated Lymphocytes with an Autologous and an Allogeneic B-Cell Line

Exp.	Donor	Stimulator	Cytotoxicity against Molt-4			Cytotoxicity against Raji		
			40:1	20:1	10:1	40:1	20:1	10:1
			^{51}Cr Release					
A	Fahey	None	19	9	2	−2	−3	−1
		Autologous line	72	65	62	5	4	1
		Allogeneic line	85	84	68	8	6	3

Lymphocytes were stimulated with an autologous and an allogeneic B-cell line for 5 days and tested for cytotoxicity against the cell lines Molt-4 and Raji.

given in which eight different lymphocyte donors were stimulated with the same allogeneic B-cell line and subsequently tested for [^{3}H]thymidine incorporation and cytotoxicity against Molt-4. With each donor there is a strong proliferative response and with all donors there is a marked increase in the capacity of the cells to kill Molt-4 except possibly with stimulated lymphocytes from donor 2.

In Table IV we have compared the capacity of allogeneic vs. autologous B-cell lines to generate Molt-4 killing. It is thus clear that generation of killer cells is not related to an expression of non-self HLA. In Table VII, experiment A and 30 and in Table VIII, experiment B, are further support of this conclusion. This finding is in line with earlier reports which also show that autologous B-cell lines act as strong stimulators in this system (9, 13).

The property to induce DNA synthesis and killer cells is mainly confined to B-cell lines (26). This is illustrated in Table V in which a slight increase of

TABLE V

Preferential Induction of DNA Synthesis and Generation of Killer Cells with SK Specificity during Stimulation of Unfractionated Lymphocytes with B-Cell Lines as Compared to Stimulation with T-Cell Lines

Exp.	Donor	Stimulator	Cytotoxicity against Molt-4			Cytotoxicity against Raji			DNA Synthesis
			12:1	6:1	3:1	12:1	6:1	3:1	
					^{51}Cr *release*				*cpm/0.1 ml culture*
16	Elwell	None	15	4	−2	−2	−1	0	1,121
		Molt-4 (T)	25	15	4	−3	−3	0	2,622
		HSB-II (T)	31	21	12	−3	−1	−4	3,187
		322 (B)	74	67	59	17	2	−3	19,759
		327 (B)	60	50	46	−3	2	−1	10,998

Lymphocytes were stimulated with two allogeneic T-cell lines, Molt-4 and HSB-II, and with two allogeneic B-cell lines, 322 and 327, and tested for cytotoxicity against Molt-4 and Raji and for [^{3}H]thymidine incorporation.

TABLE VI

Comparison between the Capacity of Allogeneic Lymphocytes Versus Autologous B Line Cells to Stimulate Generation of Killer Cells with SK Specificity from Lymphocytes Initially Depleted of SK Cells

Exp.	Donor	Stimulator	Cytotoxicity against Molt-4			Cytotoxicity against Raji		
			20:1	10:1	5:1	20:1	10:1	5:1
					^{51}Cr *release*			
B	M. Brown	Allogeneic lymphocytes	25	13	5	14	6	−1
		Autologous B-cell line	69	62	51	11	5	4
		None (unfractionated cells at day 0)	32	26	19			
		None (depleted cells at day 0)	3	0	1			

Lymphocytes initially depleted for SK cells by EAC (7S) rosette sedimentation (24) were stimulated by fresh allogeneic lymphocytes and by autologous B-cell line cells for 5 days and tested for cytotoxicity against Molt-4 and Raji cells. The proliferative response was strong in both instances as estimated by the percentage of blast transformed responder cells.

[^{3}H]thymidine incorporation and Molt-4 cytotoxicity occurs with the two T-cell lines, Molt-4 and HSB-II, whereas the B-cell lines 322 and 327 trigger a much stronger response. It may thus be tentatively concluded that the difference between B- and T-cell lines to act as stimulators is more of a quantitative than of a qualitative nature.

We then investigated whether lymphoproliferation induced by fresh allogeneic lymphocytes in a conventional mixed lymphocyte culture (MLC) would result in a generation of killer cells with SK specificity. A representative experiment is shown in Table VI from which it can be concluded that much less SK cytotoxicity is generated by allogeneic lymphocytes as compared to autologous B-cell lines. Furthermore, the MLC generated killer cells have a much lower Molt-4 specificity with an RSI of 0.44 at a lymphocyte:target ratio of 20:1 (although the Molt-4 cytotoxicity is too low to allow a correct RSI calculation

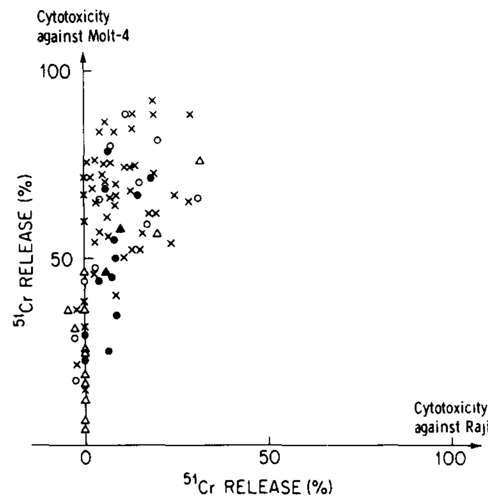

FIG. 4. Different stimulated lymphocyte subpopulations fractionated as described in Materials and Methods were tested for spontaneous cytotoxicity against Molt-4 and Raji ×, unfractionated; △, E receptor positive; ○, E receptor negative; ▲, Fc receptor positive; ●, Fc receptor negative (17 experiments).

[Materials and Methods]) compared to an RSI of 0.88 at a lymphocyte:target ratio of 10:1 for killer cells generated by the autologous B-cell line. In this experiment, killer cells were generated from virtually noncytotoxic lymphocyte populations depleted for EAC (7S) receptor-bearing cells. No test for [^{3}H]thymidine incorporation was done in this experiment, but morphologically, by making a rough estimate of the number of responding blast cells, it was clear that both the allogeneic fresh lymphocytes and the autologous B-cell lines caused a strong proliferative response.

In Fig. 4 we have summarized 17 experiments in which unfractionated, E receptor positive or E receptor negative lymphocytes or Fc receptor positive or Fc receptor negative lymphocyte subpopulations were stimulated with B-cell lines and subsequently tested for Molt-4 and Raji cytotoxicity. The purpose of Fig. 4 was to get a quantitative relationship between the Molt-4 killing and Raji killing and to compare the specificity of generated killer cells to that of unstimulated SK cells. Fig. 4 resembles essentially Fig. 3 in that the Molt-4 specificity is high below 50% of cytotoxicity and has a tendency to become less specific above 50%, and in that killer cells with a similar specificity can be generated from all cell populations, even from initially noncytotoxic Fc receptor negative lymphocytes.

The RSI of B-cell line generated killer cells from unfractionated lymphocytes have been calculated in Table VII. The mean RSI from 18 experiments was found to be 0.86 as compared to a mean RSI of unstimulated lymphocytes of 0.84 (12 experiments). It is thus clear that these two different categories of cytotoxic effector cells are very similar as far as target cell specificity goes. In Table VIII a similar RSI calculation has been done with killer cells generated from mostly noncytotoxic Fc receptor negative lymphocyte populations (except in experiments 27, 29, and 37). In seven experiments the killer cells had a mean RSI of 0.85 which means that these killer cells also have a very similar specificity to

TABLE VII

RSI and Amount Generated Cytotoxicity against MOLT-4 during Stimulation of Unfractionated Lymphocytes with B-Cell Lines

Exp.	Donor	Unstimulated		RSI	Stimulated			Generated cytotoxicity
		Ratio	Cytotoxicity		Ratio	Cytotoxicity	RSI	
			% ^{51}Cr release			*% ^{51}Cr release*		*% of total*
A	Fahey	40:1	19	?	10:1	68	0.96	97
					20:1(AS)	65	0.94	86
15	Folkert	10:1	70	0.99	10:1	75	0.99	7
16	Elwell	25:1	33	0.82	25:1	71	1.00	54
					25:1	73	0.77	77
27	Rodmar	15:1	72	0.97	3:1	85	0.85	35
28	D. Brown	40:1	35	1.00	5:1	61	1.00	93
30	Purdue	30:1	40	0.86	15:1(AS)	85	0.92	74
					7:1	86	0.85	88
33	Stein	40:1	37	0.89	10:1	68	0.96	81
35	Vesera	20:1	12	?	5:1	67	0.90	91
37	Gunnels	6:1	50	0.60	25:1	36	0.69	−100
37	Birgitta	25:1	17	?	12:1	68	0.75	84
38	Fehniger	10:1	72	0.79	5:1	62	0.69	6
38	Shope	20:1	10	?	10:1	51	0.69	93
39	Macintosh	20:1	20	?	5:1	64	0.89	100
39	Kechejian	20:1	35	0.77	5:1	79	>0.76	94
39	Flink	40:1	34	0.72	5:1	86	>0.86	98
39	Jondal	20:1	46	0.81	5:1	79	>0.69	65
42	Thiele	25:1	15	?	6:1	56	0.77	93
43	Karjala	30:1	38	0.92	15:1	71	0.92	72
				Mean: 0.84(12)			Mean: 0.86(18)	

Amount induced Molt-4 cytotoxicity is calculated as: cytotoxicity with stimulated cells minus cytotoxicity with unstimulated cells through cytotoxicity with stimulated cells and estimated by the lymphocyte:target ratio given for the stimulated cells. The relative specificity index was calculated as described in Materials and Methods.

that of SK cells. The lymphocyte donors which had unstimulated Fc receptor negative killer cells in experiments 27, 29, and 37 were subsequently retested at two separate occasions. At both times all three donors were found to have Fc negative effector cells although the amount of cytotoxicity expressed in this subpopulation, as compared to unfractionated cells, varied.

Discussion

In the present work we have taken advantage of the fact that human SK cells have a clearly defined specificity when assayed in a short-term ^{51}Cr release assay as they preferentially recognize and kill target cells of tumor origin (8 and Table I). Furthermore, we have demonstrated that this specificity is not due to a nonspecific resistance to cell-mediated lysis on the part of the SK resistant target cells as these cells were killed when Con A was present in test medium. Con A does presumably in this situation only act by bringing effector and target cells together and can thus be considered as a substitute for target

TABLE VIII
RSI and Amount Generated Cytotoxicity against MOLT-4 during Stimulation of Lymphocytes Depleted for Fc Receptor-Bearing Cells with B-Cell Lines

Exp.	Donor	Unstimulated			Stimulated			Generated cytotoxicity
		Ratio	Cytotoxicity	RSI	Ratio	Cytotoxicity	RSI	
			% ^{51}Cr release			*% ^{51}Cr release*		*% of total*
B	M. Brown	30:1	2	?	5:1(AS)	61	0.90	97
19	Thiele	25:1	9	?	25:1	39	1.00	77
24	Slaback	32:1	0	?	16:1	55	0.84	100
27	Rodmar	15:1	23	?	3:1	68	0.91	90
28	D. Brown	20:1	5	?	20:1	30	?	67
29	Drew	40:1	35	0.89	10:1	69	0.80	87
37	Gunnels	50:1	50	0.64	100:1	50	0.82	−44
37	Keld	100:1	7	?	25:1	35	0.71	94
				Mean: 0.77(2)			Mean: 0.85(7)	

Amount generated cytotoxicity was estimated as described in the legend to Table VII. The relative specificity index was calculated as described in Materials and Methods.

cell recognition. We have subsequently obtained additional evidence which clearly shows that Raji target cells or other SK resistant targer cells are not per se resistant to lysis. We have thus found that a 6-day pokeweed mitogen stimulation leads to the induction of killer cells which are equally, if not more, cytotoxic to Raji cells and other SK resistant target cells as compared to highly SK susceptible target cells such as Molt-4.[2] The implication of this finding is at present unclear. K cells and antibodies seem not to be involved either in the SK system or in the pokeweed mitogen generation system.

The major effort in the present paper has been to link the SK system to the B-cell line stimulation system in which killer cells are generated by cocultivation with autologous or allogeneic cells (9–13). It seems clear from the present work that B-cell line generated killer cells have a very similar target cell specificity as compared to that of the SK cells. We tentatively interpret this to mean that these two systems may be interconnected in the sense that antigens, similar to those expressed on Epstein-Barr virus transformed B-cell lines, may be involved in the in vivo generation of SK cells. Apart from the induction of cytotoxic effector cells with SK specificity, it is also clear that specific killer cells directed against the alloantigens of the stimulating B-cell line develop during the 5-day culture period (12).[3] In our present work we have not tested for cytotoxicity directed against alloantigens as we have been primarily interested in detecting killer cells with SK specificity. It is interesting though to note that Seeley and Golub when testing killer cells generated in conventional MLC cultures, find that effector cells cytotoxic to SK susceptible target cells appear before the specific killer cells and that they also disappear before the specific cells (27).

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[2] S. Targan and M. Jondal. 1978. Manuscript in preparation.

[3] C. S. Spina and J. L. Fahey. 1977. Specific and shared antigens on human lymphoid cell lines detected by induced T cell cytotoxicity. Manuscript submitted for publication.

The molecular basis underlying SK and B-cell line generated cytotoxicity is unclear and does not allow a comparison between the two systems. It is clear, however, that B-cell line generated killer cells do not express Fc receptors even when generated from Fc receptor positive lymphocytes. Already after short-term stimulation (48 h), no effector cells bind to immune complex monolayers and no ADCC effector cells can be dectected against antibody covered nucleated target cells.[4] It does thus appear as if an ADCC-like effector mechanism as suggested for the human SK system by Akire and Takasugi and Koide and Takasugi (28, 29) is less likely for the generated killer cells. In fact, we have to date no evidence in the SK system either that SK effector cells act by cytophilically absorbed IgG. Most of our present data does not support this hypothesis. For example, we find no inhibition of SK cytotoxicity with anti-Ig antibodies and no inhibition of SK cytotoxicity regeneration after trypsin treatment of lymphocytes by protein A, a molecule known to compete with cellular Fc receptors for IgG molecules.

From the present work, and earlier reports, it is clear that stimulatory antigens are expressed preferentially on B-cell lines and less, or not at all, on T-cell lines (26). This circumstance raises the possibility that HLA-D antigens may be involved, especially as it has recently been shown that autologous fresh B cells can be stimulatory. Opeltz et al., Kuntz et al., and Billings et al. have shown that the conventional MLC reaction can be inhibited with rabbit antisera directed against all known HLA-D allospecificities (30–32, R. Billings, personal communication). We have used the same antisera (kindly provided by Dr. Ron Billings, University of California at Los Angeles) and the same approach as Billings et al. to investigate this possibility but so far these antisera have not reproducibly inhibited the proliferative response in our system which possibly may be related to a higher antigen expression on the cell lines as compared to the fresh B cells.

Even if we at the present time have no evidence that B cell associated alloantigens are responsible for the proliferative response it is interesting to note that there is a similarity between the present system and the MLC reaction in the sense that the stimulation antigens and the target antigens are distinct. In the MLC reaction HLA-D serves as inducer of proliferation but not as target resembling the inverse relationship between ability to stimulate and susceptibility to cytotoxicity in the present system. Thus, B-cell lines of normal origin are good stimulators but poor targets in contrast to T-cell lines which are good targets but poor stimulators. This distinction has also quite recently been noted by Vande Stouwe et al. who found less killing of the stimulating cell line as compared to unrelated cell lines (33).

If SK cell generation is linked to B-cell associated "amplification" antigens, this would advance our knowledge about the nature of the SK system. For one thing, it is clear that these antigens are also expressed on cells belonging to the monocyte-macrophage series (34), and it is thus possible that these non-B cells are also involved in SK cell regulation. We are presently investigating this

[4] S. Targan and M. Jondal. 1978. Lack of Fc receptor expression on cytotoxic effector cells with spontaneous killer (SK) cell specificity induced by stimulation with B cell lines. Manuscript submitted for publication.

possibility. Another interesting point is that Svedmyr has shown that the proliferative response to autologous B-cell lines involves a short memory function as it is reduced during restimulation and absent during a second restimulation (12). This was in contrast to the T-cell response in conventional allogeneic MLC cultures in which responding cells became increasingly more active when repeatedly stimulated during a 4-mo period (12). If the proliferative response is involved in the generation of SK killer cells, it would thus appear that memory function in the SK system is short-term and that MLC responsive T cells and SK cells belong to different cell populations. In this context it is also interesting to note that we were able to induce killer cells from all tested subpopulations (Fig. 4 and Table VIII) even from Fc receptor negative non-SK cells. In addition to the induction of cytotoxic effector cells, we also found a strong proliferative response as measured by [^{3}H]thymidine incorporation in all tested subpopulations except in SRBC receptor negative lymphocytes (as reported in more detail elsewhere).[4] With SRBC receptor negative cells, which also are unresponsive to several mitogens (35), we furthermore found that B-cell line stimulation did increase the cytotoxic potential although the lack of proliferation correlated with the retention of Fc receptors on the effector cells.[4] The capacity of all subpopulations to respond to B-cell line stimulation by induction of SK cytotoxicity demonstrates an impressing potential of the system.

In summary, our results show that B-cell associated antigens can cause the induction of cytotoxic effector cells which have SK specificity. Furthermore, both the preformed nature of the SK system and the short memory function in the B-cell line response as compared to a conventional T-cell response may indicate a function for the SK system as a primary defense barrier in the immune response which has the capacity to act before a more conventional immune response is mounted. It is easy to conceive that there must be a great advantage for the host to have a lymphocyte cell population with a preformed "spontaneous" specificity which can deal with a low number of target cells, whether virus infected or transformed in nature, before these multiply to such a level that they can induce a B- and T-cell response. Finally, if the SK system is at all important as an anti-tumor surveillance system, one may consider the possibility to activate SK cells in vivo. If our results are taken to demonstrate in vitro SK activation, these may eventually be extended to allow a similar activation in vivo.

Summary

The present work shows that stimulation of peripheral blood lymphocytes with autologous or allogeneic B-cell lines leads to a strong induction of cytotoxic effector cells with spontaneous killer (SK) cell specificity, apart from the specific response directed against the particular stimulating cell. To demonstrate this we have determined a relative target cell specificity in the SK system, defined by the short-term ^{51}Cr release assay, and established a relative specificity index (RSI). Using this approach we have been able to show that killer cells induced during a 5-day cocultivation period with B-cell lines have a similar PSI to that of unstimulated SK cells. In addition, we have shown that such killer cells can be induced from several different lymphocyte subpopulations and that they, in

contrast to SK cells, do not express Fc receptors. The implications of these findings in relation to the nature, mechanism, and biological significance of the SK cell system is discussed.

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