"ANOMALOUS" THY-1$^+$ KILLER CELLS IN ALLOGENEIC AND F_1-ANTI-PARENTAL MIXED LEUKOCYTE CULTURE

Relation to Natural Killer Cells and Allospecific Cytotoxic T Lymphocytes*

By KLAS KÄRRE, JANET K. SEELEY,‡ ERENE ERIKSSON, ROBERT C. BURTON,§ AND ROLF KIESSLING

From the Department of Tumor Biology, Karolinska Institutet, S-104 01 Stockholm, Sweden

Our present understanding of the mechanisms involved in activation, specificity, and restriction of T cells is largely indebted to studies of cytotoxic responses of lymphocytes in vitro. During the course of such studies, several investigators (1–5) have observed generation of effector cells with the capacity to kill autologous as well as allogeneic cells without any apparent specificity or restriction in relation to the sensitizing antigens/cells. Such phenomena have frequently been dealt with in terms of T cell responses, with "promiscuous cytotoxicity" (1) or "specificity for self H-2" (2, 4). More recently, however, this type of culture-induced reactivity has been discussed more in relation to natural killer (NK)[1] cells (5–11), even if the responsible effectors in question express Thy-1 antigen and do not arise in cultures prepared with spleen cells from nude mice (4, 12). These two interpretations are not necessarily contradictory: although many investigators stress the distinction between the NK and T cell systems (13, 14), others are more impressed by the similarities between them, especially at the effector cell level (15, 16). According to one unifying hypothesis (16), killing by "specific cytotoxic T lymphocytes (CTL)," "NK cells," and "nonspecific effector cells activated in culture" are all different functions mediated by largely overlapping effectors in the T lineage, the former (CTL function) being dependent on a specific receptor system recognizing H-2 alone or as restriction element in connection with antigen, and the two latter (seemingly nonspecific functions) based on an as yet undefined membrane property enabling the binding to and killing of certain "sensitive" cells.

We have previously described (10) a cytotoxic response against NK-sensitive

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[1] *Abreviations used in this paper:* AK, anomolous killer, anomalous killing; AT × FL, adult thymectomy, irradiation, and reconstitution with fetal liver cells; BSA, bovine serum albumin; Con A, concanavalin A; CTL, cytotoxic T lymphocyte; FCS, fetal calf serum; IFN, interferon; IL-2, interleukin 2; LCMV, lymphocyte choriomeningitis virus; MLC, mixed leukocyte culture; NC cell, NK 1$^-$,Asialo GM 1$^-$ natural cytotoxic cell; NCS, newborn calf serum; NK, NK 1$^+$, Asialo GM 1$^+$ natural killer cell; NMS, normal mouse serum; NW, nylon wool; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; RC, rabbit complement; SBA, soybean agglutinin.

semisyngeneic and xenogeneic tumor cell lines occurring early in the murine mixed leukocyte culture (MLC) combination (CBA × A/Sn)F_1→C57B1. The effector cells in this system, which were characterized as Thy-1.2-positive blasts, are one example of nonspecific effector cells activated in culture described above. These anomalous killer (AK) cells represent a striking analogy to nonspecific Thy-1^+ killer cells elicited in the response to certain virus infections in vivo (10). In the present study, we investigated the relation between anomalous killing and the phenomena of natural as well as specific CTL killing. The results indicate that MLC-generated AK cells are derived from NK cells, which have been activated and have undergone surface marker modulation under the influence of antigen-stimulated T cells.

Materials and Methods

Animals. Mice of the following inbred strains and F_1 hybrids were bred in our own animal colony: CBA, A/Sn (A), C57B1/6 (C57B1), C57B1 +/bg^J, C57B1bg^J/bg^J (C57B1 *bg/bg*), DBA/2 (DBA), (CBA × A)F_1, and (A × C57B1)F_1. BALB/c, BALB/c *nu/nu*, BALB/c +/*nu*, and C57B1 *nu/nu* mice were purchased from Gl. Bomhotgård Ltd., 8860 Ry, Denmark. Random-bred Swiss +/*nu* and *nu/nu* littermates were a kind gift from Dr. Beppino Giovanella, The Stehlin Foundation for Cancer Research, Houston, TX.

Adult thymectomy. Surgical thymectomy was performed on 3–4-wk-old, anesthesized animals. 3 wk later, the thymectomized mice and control litter mates received 650 rad whole-body irradiation and were reconstituted by intravenous injection of 5–15 × 10^6 syngeneic fetal liver cells. Mice treated with adult thymectomy, irradiation, and fetal liver reconstitution (AT × FL) according to this protocol did not give antibody responses (titers < 1/10) after challenge with the thymus-dependent antigen horse erythrocytes, whereas control mice (C × FL) gave titers > 1/80 when tested in a hemolysis assay 3–4 wk after reconstitution (unpublished observation).

Tumors. YAC-1 is a Moloney virus induced lymphoma derived from the A/Sn strain. YAC-ACG, YAC-ACK, and YAC-8 are variant sublines of YAC-1, which have been selected for low NK sensitivity by repeated exposures to highly efficient NK cells from CBA × A mice in vitro and in vivo. The procedure and the variant sublines have been described in detail in a recent report (17). RBL-5 is a Rauscher virus-induced C57B1 lymphoma. MPC-11 (mineral oil-induced plasmocytoma), RL-♂ (radiation-induced lymphoma), and WEHI-164 (methylcholanthrene-induced fibrosarcoma) are all derived from the BALB/c strain. The cell lines were maintained as suspension cultures in F-13 medium supplemented with penicillin, streptomycin, and 10% fetal (FCS) or newborn calf serum (NCS). This medium was also used for preparations of cells as well as in cytotoxicity and competition assays.

Preparation of Cells and Cultures. Single-cell suspensions of lymph node or spleen cells were prepared in F-13 NCS. After washing and counting, cells were used either directly in a cytotoxic assay (day 0, fresh spleen cells) or for cultures: 20–30 × 10^6 (usually 25 × 10^6) cells were plated alone or with 6–15 × 10^6 (usually 10 × 10^6) irradiated (2,000 rad) stimulator cells in 50-ml Falcon tissue culture flasks (Falcon Labware, Oxnard, CA). Thus, the final responder/stimulator ratio in MLC varied between 2:1 and 3:1, which yielded similar results. Culture medium (RPMI with penicillin-streptomycin, 3 × 10^{-5} M 2-mercaptoethanol, and 10 mM Hepes buffer) supplemented with 10% FCS was added to a final volume of 10 ml, and the cultures were incubated at 37°C, 5% CO_2.

In some experiments, cultures were set up in Click's medium (18) supplemented with 0.5–1% FCS or fresh (CBA × A)F_1 normal mouse serum. The most extensively used MLC combination was (CBA × A)F_1 responders against irradiated C57B1 stimulator cells, denoted (CBA × A)F_1→C57B1_x.

For mitogen stimulation, triplicate aliquots of 2 × 10^5 cells were incubated in a 0.15-ml volume supplemented with 10% human AB-serum and the mitogen (phytohemagglutinin (PHA): 10 μg/ml, concanavalin A (Con A): 7.5 μg/ml) in flat-bottomed wells in Falcon microtiter plates. After 72 h, each well was pulsed with 0.5 μCi of [^{3}H]thymidine and incubated further for 6 h. The cells were harvested onto strips of fiber glass filter paper, dried, and the

individual samples were placed in liquid scintillation fluid for counting in a scintillation counter.

Cytotoxicity Assay. 100μCi of ^{51}Cr was added to 4×10^6 target cells in 0.3 ml of F-13-NCS and incubated at 37°C for 45 min. Subsequently, the cells were washed three times and suspended to a concentration of 2×10^5/ml. 1×10^4 cells/50 μl were added to the log-titered effectors in a total volume of 150 μl in Linbro microtest plates (Linbro Chemical Co., Hamden, CT). The plates were incubated 3–6 h at 37°C in 5% CO_2, centrifuged at 1,200 rpm for 3 min, and then 75 μl supernatant was collected and counted in a gammaspectrometer (Beckman Instruments, Fullerton, CA). The spontaneous release was determined by adding target cells to medium alone. The spontaneous release did not exceed 20% unless otherwise stated.

The percentage of lysis was calculated according to the following formula: ([test counts − spontaneous release]/[total counts − spontaneous release]) × 100. Variation of triplicate samples was < 15% of the mean, when lysis exceeded 10%. Cytotoxicity of cultured cells below 10% lysis was not considered significant.

Competition Assay. Unlabeled tumor cells were incubated at four different concentrations (24×10^4, 12×10^4, 6×10^4, and 3×10^4) with a constant number of effector cells (2×10^5) for 30 min at 37°C. 1×10^4 ^{51}Cr-labeled target cells were then added to a final volume of 0.15 ml as described for cytotoxic assay, and triplicate samples were incubated for 6 h. Thus, a constant effector/target cell ratio of 20:1 was used in most competition experiments while the number of competing unlabeled tumor cells were titrated out. The results are expressed as percent inhibition of specific lysis in control samples (effectors and labeled targets without competitors, which yielded 17 to 35% specific lysis).

Separation of Soy Bean Agglutinin-positive (SBA^+) and SBA^- Cells. The preparation of SBA was a kind gift from Professor Nathan Sharon, The Weizmann Institute of Science, Rehovot, Israel. For separation of spleen cells according to their expression of SBA-receptors, 2×10^8 splenocytes were resuspended in 0.5 ml SBA in phosphate-buffered saline (PBS) (2 mg/ml). The cells were left at room temperature for 5 min with occasional shaking to allow for agglutination of SBA^+ cells. A 0.25-ml volume of the cell suspension was then gently layered on top of 10 ml bovine serum albumin (BSA) diluted to 2.5% in PBS in a 15-ml tube. The SBA^+ cell formed large agglutinates which sedimented rapidly, whereas the single SBA^- remained on top of the BSA-PBS. After 10 min of sedimentation, the top (SBA^-) and bottom (SBA^+) fractions were collected with a pasteur pipette. The two fractions and the control cells (the remaining 0.25 ml volume, which had been mixed with the BSA-PBS without separation) were then washed twice in 0.2 M D-galactose in PBS to allow the monosaccarides to compete specifically for SBA-receptor sites, thereby disassociating the agglutinates and washing away the lectin. The cells were then washed three times in RPMI-FCS before functional testing or culture.

Removal of Adherent and Phagocytic Cells. $500–750 \times 10^6$ cells were incubated on a washed and prewarmed nylon wool (NW) (type 200; Fenwal Laboratories, Deerfield, IL) column in a 50-ml syringe at 37°C in 5% CO_2. After 20 min, the cells were eluted and again incubated on a similar column for 45 min. In some experiments, the cells eluted from the second column (NW × 2) were incubated with carbonyl iron in RPMI-NCS (2×10^6 cells/ml) in 50-ml tissue culture flasks at 37°C. After 40 min, the iron-containing cells were removed by decanting the suspensions with a strong magnet applied to the surface of the flask (NW × 2 + Fe/Magnet).

Treatment with Alloantisera Plus Complement. Hyperimmune alloantisera were produced by repeated intraperitoneal immunizations (five at the minimum) of $(DBA \times C57B1)F_1$ mice with CBA spleen, liver, and kidney (anti $H\text{-}2^k$), and CE or $(CE \times NZB)F_1$ mice with CBA spleen (anti-NK 1.2 [11, 19]), followed by bleeding, collection, and inactivation of serum. Anti $H\text{-}2D^b$ serum [$(B10.A(5R) \times LP.R.III)F_1$ anti-B10, produced by a similar protocol using thymus, spleen and lymph node for immunizations] was kindly provided by the National Institutes of Health Resources Branch. Monoclonal anti-Thy-1.2 (lyophilized ascites from the F7D5 IgM producing hybridoma) (20) was a kind gift from Dr. P. Lake and Dr. E. A. Clark, then at University College Hospital Medical School, London.

For specific depletion, fresh or culture-derived cells were incubated at a concentration of 10^7/ml in antiserum or control serum (FCS or NMS) diluted in RPMI in 37°C, 5% CO_2. After 30 min, cells were pelleted and resuspended in a 1/10 dilution of a selected rabbit complement, in the same volume as used for the incubation of antiserum. The cells were incubated for a

further 40 min and then washed three times in RPMI-NCS. If the suspension was to be used for culture, viable cells were counted to equalize the number of plated cells. If the cells were to be used for a cytotoxicity assay, they were resuspended in the original volume (corresponding to 10^7 cells/ml before treatment) without equalizing cell concentrations. A 0.1-ml volume from this suspension was added to target cells in the cytotoxicity assay. Thus, the total remaining effector cell potential was compared for control and antiserum-treated cells.

For calculation of percent functional reduction (of mitogen responsiveness or cytotoxicity) of the antiserum-treated cells, the following formula was used: 100 − [([^{3}H]thymidine cpm of (or % lysis mediated by) antiserum treated cells)/([^{3}H]thymidine cpm of (or % lysis mediated by) control serum treated cells)] × 100. Note that the percent reduction is only a semiquantitative reflection of the proportion of cytolytic or mitogen-responsive cells that were killed by the antiserum treatment.

Interferon Treatment. The partially purified β interferon (IFN-β) preparation (produced by infection of L cells with Newcastle disease virus, sp. act. 2.2×10^7 U/mg/ml, 0.053 mg protein/ml) was purchased from Calbiochem-Behring Corp., (La Jolla, CA). YAC-1 cells were incubated at 0.5×10^6 cells/ml in RPMI-FCS with 5×10^3 U of IFN-β/ml for 16 h (21). Control samples of YAC-1 cells without IFN were incubated in parallel. The cells were washed twice before labeling with ^{51}Cr.

Results

Kinetics and Specificity of Anomalous Killing Generated in Different Types of MLC. MLC in autologous mouse serum: (CBA × A)F_1→C57B1$_x$MLC in Click's medium with 0.5–1% autologous (CBA × A) mouse serum (NMS) yielded an anti YAC-1 response peaking by day 4–5, shortly before the allospecific killing (Table I). The kinetics of both responses were thus somewhat delayed in comparison with MLC in FCS (10). Control cultures without stimulator cells in NMS showed no signs of blastogenesis or proliferation and yielded a very poor recovery of lymphocytes (<5%) which gave

TABLE I

Kinetics of Cytotoxic Responses in Different Types of MLC

Experiment	Responders	Stimulators	Serum	Percent lysis*						
				YAC-1				RBL-5		
				Day 1 (24 h)	Day 3–4 (60–90 h)	Day 5 (100–120 h)	Day 6–7 (120–140 h)	Day 3–4	Day 5	Day 6–7
1	(CBA × A)F_1	—	1% NMS‡	0	0	0	0	0	0	0
	(CBA × A)F_1	C57B1	1% NMS‡	10	9	24	9	0	49	74
2	(CBA × A)F_1	—	1% FCS‡	6	9	3	0	0	3	3
	(CBA × A)F_1	C57B1	1% FCS‡	16	26	5	0	8	46	37
3	(CBA × A)F_1	—	10% FCS	19	11	20	—§	—	9	—
	(CBA × A)F_1	CBA × A	10% FCS	18	14	12	—	—	4	—
	(CBA × A)F_1	A	10% FCS	14	15	20	—	—	6	—
	(CBA × A)F_1	C57B1 *+/bg*	10% FCS	30	50	26	—	33	71	—
	(CBA × A)F_1	C57B1 *bg/bg*	10% FCS	23	49	29	—	39	74	—
	(CBA × A)F_1 1,000 rad	C57B1 *+/bg*	10% FCS	8	4	0	—	0	0	—
4	(CBA × C57B1)F_1	—	10% FCS	12	12	15	—	3	2	—
	(CBA × C57B1)F_1	C57B1	10% FCS	16	36	25	—	3	5	—
5	(A × C57B1)F_1	—	10% FCS	—	4	5	—	—	—	—
	(A × C57B1)F_1	C57B1	10% FCS	—	16	9	—	—	—	—

* Effector/target ratio was 50:1, except in experiment 3, where it was 100:1.

‡ In Click's medium.

§ Not tested.

background values (<5% lysis) when used as effector cells against YAC-1 targets (Tables I and II).

MLC in FCS. In contrast to the control cultures in NMS, spleen cells kept without allostimulation in 1–10% FCS regularly showed variable degrees of blastogenesis and cytotoxic activity. Some FCS batches tested during the course of this study were highly stimulatory and yielded strong cytotoxic responses against a variety of cell lines (not shown). Such batches were not used in the experiments in this report, for which FCS with low or moderate blastogenic activity (when used at 10% in control cultures) were selected. In some of the experiments, the anti-YAC-1 activity in these control cultures showed a regeneration after the initial decline during the first 24 h, with the peak occurring between days 3 and 6 (i.e., experiment 3 in Table I), whereas in others, it remained low throughout the culture period (experiments 2 and 5 in Table I). When C57B1 allostimulator cells were added, there was always a regeneration of YAC-1 killing, peaking between 60 and 90 h (day 3–4), previously termed anomalous killing (10) (Table I). The cytotoxic potential against YAC-1 at this time point varied between experiments, but was always stronger in MLC (15–60% lysis) than in control cultures (0–20% lysis). In some (but not all) experiments, the activity of allostimulated cells was somewhat higher than that of control cells already after 24 h of culture (Table I). Thus, whereas there were sometimes small differences in YAC-1 killing between effectors from MLC and control cultures by days 1 and 5, the largest difference and strongest anomalous killing was always seen around day 3–4. For the further experiments in this study, cultures were routinely performed in 10% FCS, unless otherwise indicated.

Direct Killing and Cold Target Inhibition with NK-sensitive Tumor Cells and Effectors from

TABLE II

Direct Cytotoxicity and Cold Target Inhibition with NK-sensitive Tumor Cells and AK Cells from Different Types of MLC

Experiment	Responders	Stimulators	Serum	Percent lysis*			Percent cold target inhibition						
				Target	0 h	70–100 h	Inhibitor cells	YAC-1 targets			RBL-5 targets		
								3:1‡	6:1	12:1	3:1	6:1	12:1
1	(A × DBA)F$_1$	—	10% FCS	YAC-1	—	5			—			—	
	(A × DBA)F$_1$	C57B1	10% FCS	YAC-1	25	40	YAC-1	80	85	90	19	16	16
				MPC-11	10	30	MPC-11	70	75	80	21	19	16
				P815	0	5	P815	10	0	20	20	19	8
				RBL-5	0	20	RBL-5	0	10	20	40	60	100
2	(A × CBA)F$_1$	—	0.5% NMS§	YAC-1	—	4	—		—			—	
	(A × CBA)F$_1$	C57B1	0.5% NMS§	YAC-1	—	15	YAC-1	75	100	100	0	0	25
				RBL-5	—	52	RBL-5	0	0	0	35	50	70
3	BALB/c	—	10% FCS	YAC-1	—	20	—		—			—	
				RL♂	—	5	—		—			—	
	BALB/c	DBA	10% FCS	RL♂	—	14	—		—			—	
				P815	—	3	—		—			—	
	BALB/c	C57B1	10% FCS	YAC-1	26	39	—		—			—	
				RL♂	9	20	—		—			—	
				P815	0	6	—		—			—	
				RBL-5	—	70	—		—			—	

* Effector/target ratio was 50:1.

‡ Inhibitor/target ratio. Effector/target ratio was 20:1.

§ In Click's medium.

Different MLC Responder/Stimulator Combinations. To estimate to what extent radioresistant NK cells among the stimulators contributed to the YAC-1 killing observed after 3–4 d in MLC, we used C57B1 *bg/bg* splenocytes (deficient in NK activity) as irradiated allogeneic cells to sensitize (CBA × A)F_1 responders. These cultures had slightly lower NK activity after 24 h of culture than MLC with C57B1 +/*bg* stimulators (Table I). However, there was no difference between the cultures with respect to anomalous YAC-1 killing by 75 h or allospecific killing against RBL-5 (Table I). When the responder (CBA × A)F_1 spleen cells were irradiated before MLC, there was only low recovery of lymphocytes without any signs of blast transformation by day 3–4 (not shown), and without cytotoxic potential (Table I). Thus, induction of AK required nonirradiated responder cells, and was not dependent on remaining NK activity among irradiated stimulator cells.

The higher cytotoxic activity in MLC was not merely due to higher cell density in these cultures, as (CBA × A)F_1 cells cultured together with irradiated autologous (CBA × A)F_1 or semisyngeneic A (F_1→parental MLC) cells showed the same low cytotoxicity as responder cells cultured without irradiated stimulator cells (Table I). However, in certain other F_1 anti-parental MLC combinations [(CBA × C57B1)F_1→C57B1x and (A × C57B1)F_1→C57B1x)], a reproducible anomalous killing response peaking at day 4 was observed (Table I). Anomalous killing against NK-sensitive tumors was also observed in several different allogeneic combinations apart from (CBA × A)F_1→C57B1x, including (A × DBA)F_1→C57Blx. BALB/c→C57B1x and the Mls disparate BALB/c→DBA (Table II).

We have previously shown that AK cells from day 3 (CBA × A)F_1→C57B1 MLC lyse a panel of semisyngeneic or xenogeneic tumor cell lines in the same preferential order as fresh (CBA × A)F_1 NK cells (10). This NK-like target selectivity was here confirmed for AK derived from MLC using (A × DBA)F_1 or BALB/c splenocytes as responder cells. After an initial decline to <10% lysis during the first 24 h of culture (not shown), allostimulated responder cells had regenerated cytotoxicity against the highly NK-sensitive YAC-1 by day 3 (Table II). The intermediary NK-sensitive MPC-11 or RL♂ were lysed somewhat less efficiently by the same effectors, whereas no killing of the relatively NK-resistant P815 was observed. Furthermore, we could here demonstrate the NK-like target selectivity also in cold target inhibition experiments: nonlabeled MPC-11 cells, but not P815 cells, competed with MLC-induced YAC-1 killing. No cross competition with the allospecific killing against RBL-5 was seen, regardless of the type of serum used for the MLC (10) (Table II).

Direct Killing and Cold Target Inhibition with NK-resistant YAC Cells. A similar pattern of specificity emerged in tests with YAC-1 cells with low NK sensitivity, obtained by phenotypic modulation or variant selection. Thus, IFN-β pretreatment of YAC-1 cells, which results in "protection" against NK-lysis but not against anti-H-2^a-directed allospecific CTL (21), clearly decreased sensitivity to AK (Table III). The previously described YAC-1 variants selected for resistance to NK-lysis (17) also had a decreased sensitivity to AK (Table III). This may depend on reduced expression of NK recognition sites on these variants rather than a general resistance to lysis, as their capacity to inhibit YAC-1 lysis by NK or AK cells was also reduced, and they were lysed by allospecific CTL at least as efficiently as YAC-1 (Table III) (17). However, the results from direct killing as well as cold target inhibition assays indicate that the

TABLE III

Specificity of AK; Direct Cytotoxicity and Cold Target Inhibition with NK-resistant YAC-1 Cells

	Percent lysis						Percent inhibition of YAC-1 lysis	
	Fresh spleen		(CBA × A)F1 → C57B1$_x$* MLC		C57B1 → A$_x$‡ MLC		Fresh spleen	(CBA × A)F$_1$ → C57B1$_x$* MLC
	100:1	33:1	100:1	33:1	33:1	20:1		
YAC-1§	24‖	18	24‖	10	46	16	70	70
YAC-1 IFN-β¶	1	1	0	0	53	20	—	—
YAC-ACK	2	2	7	2	—	16	3	24
YAC-ACG	2	2	5	4	—	23	7	29
YAC-8	4	0	2	0	—	25	23	12
RBL-5	—	—	—	—	—	—	—	11

* MLC effectors harvested after 72 h (day 3).

‡ MLC effectors harvested after 120 h (day 6); right column represent data from ref. 17, included here for comparison. These experiments were performed with the same cultures of variant lines.

§ Tumor cells were tested as target cells in a 5-h ^{51}Cr-release assay, or as cold target inhibitor cells with ^{51}Cr-labeled YAC-1 cells. Inhibitor/target cell ratio was 12:1.

‖ Percent lysis with the corresponding effectors at 24 h and the nonstimulated effectors at 72 h were both <5%.

¶ See Materials and Methods for details.

TABLE IV

Treatment of Effector Cells with Alloantisera or Monoclonal Anti Thy 1.2 Antibody + RC

Experiment	Treatment of effector cells	C57B1 (fresh)			(CBA × A)F$_1$ (fresh)			(CBA × A)F$_1$ → C57B1 × MLC (80 h)					
		YAC-1*			YAC-1			YAC-1			RBL-5		
		1:1‡	1:2	1:4	1:1	1:2	1:4	1:1	1:2	1:4	1:1	1:2	1:4
1	FCS§ + RC	22	20	12	24	16	10	26	18	12	—	—	—
	Anti H-2^b + RC	5	2	1	28	17	11	24	18	12	—	—	—
	Anti H-2^k + RC	22	21	11	8	7	6	11	8	7	—	—	—
	Anti Thy 1.2 (100 μg/ml) + RC	—	—	—	22	16	10	10	6	6	—	—	—
2	FCS + RC	—	—	—	29	19	12	33	25	18	24	17	9
	Anti Thy 1.2 (100 μg/ml) + RC	—	—	—	24	16	9	8	4	3	−1	0	0
	Anti Thy 1.2 (10 μg/ml) + RC	—	—	—	30	19	10	13	9	5	1	−1	0

* 10^4 target cells per well in a 5 h ^{51}Cr-release assay.

‡ Original cell concentration was 10^7/ml. After treatment with serum + RC, cell suspensions were restored to original volume (1:1) and serial dilutions were performed (1:2 and 1:4); 100 μl effector cells were added to each well.

§ FCS and alloantisera used at a dilution of 1:15.

YAC-ACK and YAC-ACG were both somewhat better recognized by AK than by NK cells.

Alloantigen Surface Markers on AK Cells. Treatment of effector cells with anti-H-2D^b antiserum according to a protocol that reduced NK activity of C57B1 spleen cells by 80% did not affect the anomalous killing induced in (CBA × A)F$_1$→C57B1x cultures (Table IV). In contrast, (DBA × C57B1)F$_1$ anti CBA antiserum (containing high titers of anti H-2^k antibodies) + rabbit complement (RC) reduced the anomalous killing and NK activity mediated by fresh (CBA × A)F$_1$ spleen cells with comparable efficiency (Table IV). Thus, the vast majority of anomalous killers in our MLC

combination express the alloantigens of the responder cells, indicating that they are derived from this population and not from the irradiated stimulator cells.

Treatment with CE or (CE × NZB)F_1 anti-CBA antiserum + RC, containing high titers of anti-NK 1.2 antibodies (11, 19) was efficient in reducing NK-activity of (CBA × A)F_1 spleen cells (Table V). The same treatment caused no or only partial reduction of anomalous killing induced in allogeneic or F_1 antiparental MLC, and allospecific killing was not affected at all (Table V).

In our previous study, we found that AK cells, but not fresh NK cells, were sensitive to treatment with monoclonal F7D5 anti-Thy-1.2 antibody + RC (10). However, we were not able to abrogate AK completely by anti-Thy-1.2 treatment. Furthermore, it has now been reported by several groups independently (21–24) that a proportion of NK cells do express low density of Thy-1 antigen, detected by monoclonal reagents. We therefore tested the susceptibility of freshly or MLC-derived effector cells to the anti-Thy-1.2 reagent over a wide range of its titration curve. As seen in Fig. 1, the anti-Thy-1.2 ascites preparation killed virtually all thymocytes at a concentration of 1 μg/ml, as evaluated by trypan blue exclusion after a two-step procedure with a selected rabbit serum as source of complement. The same concentration of antibody also killed 30% of spleen cells, with the remaining population showing intact NK activity, but no proliferative response to Con A or PHA. However, a slight reduction of NK activity (10–25%) was seen when higher concentration of the ascites preparation was used (0.01–10 mg/ml). With anomalous killers, reduction was marked (70%) already with 10 μg/ml and complete with 1 mg/ml. Allospecific CTL were even more sensitive (70% reduction with 1 μg/ml). This difference in susceptibility to anti-Thy-1.2 treatment between anomalous and allospecific killers was much smaller than that between AK and NK, although it was observed consistently (Table IV).

TABLE V

Treatment of Effector Cells with Anti-NK-1.2 Antiserum + RC

Experiment	Responder genotype	Treatment of effector cells*	Percent lysis‡		
			Fresh spleen cells	Spleen cells cultured with C57B1 stimulator cells 80 h	
			YAC-1	YAC-1	RBL-5
1	(CBA × A)F_1	FCS 1:30 + RC	29	—	—
		Anti-NK 1.2§ 1:40 + RC	4	—	—
2	(CBA × A)F_1	NMS 1:30 + RC	—	24	28
		Anti-NK 1.2§ 1:40 + RC	—	19	25
3	(CBA × A)F_1	FCS 1:40 + RC	35	53	49
		Anti-NK 1.2‖ 1:40 + RC	7	36	48
4	(CBA × A)F_1	FCS 1:20 + RC	15	17	—
		Anti-NK 1.2‖ 1:20 + RC	2	14	—
		Anti-NK 1.2‖ 1:40 + RC	3	13	—
5	(CBA × C57B1)F_1	FCS 1:30 + RC	25	25	<5
	F_1 antiparental MLC	Anti NK 1.2 1:30‖ + RC	6	21	<5

* Original cell concentration was 10^7/ml. After treatment with serum + RC, cell suspensions were restored to original volume and 100 μl was added to 10^4 targets.

‡ 10^4 target cells per well in a 4–5-h ^{51}Cr-release assay.

§ CE anti-CBA antiserum.

‖ (CE × NZB)F_1 anti-CBA antiserum.

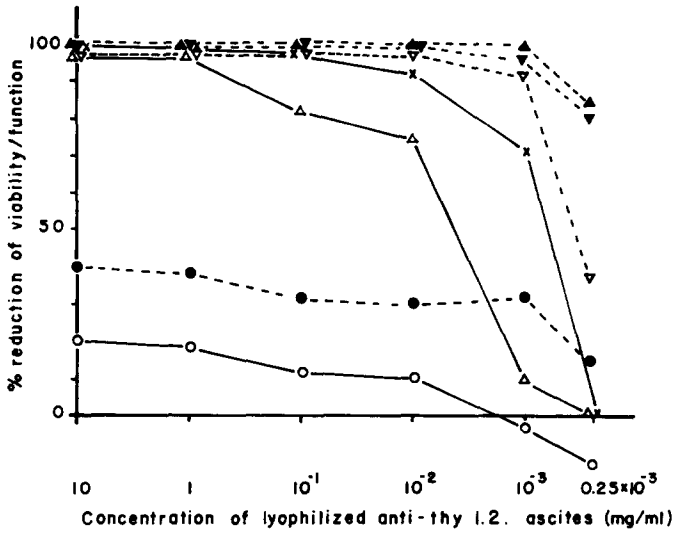

FIG. 1. Titration of monoclonal anti-Thy-1.2 antibody in an RC-dependent assay on splenocytes, thymocytes, and MLC-derived effector cells. The diagram shows the proportion of dead (CBA × A)F_1 (freshly derived) spleen cells (●) and thymocytes (▽) as judged by trypan blue exclusion; the percent reduction of responsiveness to PHA (▲) and Con A (▼) of fresh (CBA × A)F_1 splenocytes, as measured by [^{3}H]thymidine incorporation by 72 h; the percent reduction of (CBA × A)F_1 fresh splenocyte-mediated anti-YAC-1 cytotoxicity (NK [○]); and the percent reduction of MLC-derived effector cell [(CBA × A)F_1→C57B1$_x$, 80 h]-mediated anti-YAC-1 (AK [△]) and anti-RBL-5 (CTL [x]) cytotoxicity. Percent reduction was calculated by comparison to control cells (see Materials and Methods) treated with FCS (1:50) + RC.

Cellular Requirements for Induction of Anomalous Killing. Our results obtained with preirradiated responder cells and with treatment of effector cells with anti H-2 antiserum + RC demonstrated that AK derived from precursors present in the nonirradiated responder population in the MLC. It was therefore possible to investigate the nature of these precursors by selection or experimental manipulation of the responder cells.

Induction of AK did not require adherent or phagocytic cells in the responder population (Table VI). However, if the corresponding cells were removed from the stimulator population, no AK was observed day 4 (Table VI), and it was still not detectable by day 6 (data not shown). The allospecific response was also reduced by this treatment of the stimulator cells, as previously reported (25).

SBA binds to and agglutinates NK cells and B cells, but not mature T cells, which lack the receptor for this lectin (26).[2] Responder spleen cells depleted of NK and B cells by agglutination with SBA gave a poor AK response, although they yielded an equally efficient allospecific CTL response as compared with control spleen cells, which had been incubated with the lectin without separation of SBA^+ and SBA^- cells (Table VII). Lymph node cells from the same group of animals gave a similar picture: they were less efficient in NK lysis day 0, showed no or little AK, yet produced a highly efficient allospecific CTL response (Table VII).

These results indicated that the AK potential of the responder population might depend on its content of NK cells. In further support of this, we found that treatment

[2] Kärre, K., Y. Reisner, and N. Sharon. Manuscript submitted for publication.

TABLE VI

Induction of AK Requires Adherent Cells in the Stimulator Population

Responder cells*	Stimulator cells	Percent lysis	
		YAC-1 targets‡	RBL-5 targets
(CBA × A)F_1-unfractionated	—	8	—
(CBA × A)F_1-unfractionated	C57Bl-NW × 2 + Fe/Magnet‡	6	—
(CBA × A)F_1-unfractionated	C57Bl-unfractionated	18	—
(CBA × A)F_1-NW × 2 + Fe/magn	C57Bl-unfractionated	57	—
(CBA × A)F_1-NW × 2	—	0	1
(CBA × A)F_1-NW × 2	C57Bl-NW × 2	1	9
(CBA × A)F_1-NW × 2	C57Bl-unfractionated	15	34

* Spleen cells. Effector cells were harvested from 80 h cultures and tested at an Effector/target ratio of 100:1. By 24 h, the cells from the corresponding cultures gave <6% lysis.
‡ See Materials and Methods.

TABLE VII

Poor AK Response with Responder Cells from Lymph Node and the SBA^- Spleen Cell Fraction

Responder cells*	Stimulator cells	Percent lysis‡			
		YAC-1 targets			RBL-5 targets‡
		0 h	24 h	72 h	120 h
Total spleen population§	None	30	12	4	—‖
	C57Bl spleen		12	*26*	65
SBA^- spleen cells¶	None	7	4	1	—
	C57Bl spleen		4	4	70
Lymph node cells	None	5	3	1	—
	C57Bl spleen		5	3	65

* All responder cells in this table from the same group of (CBA × A)F_1 mice.
‡ Effector/target ratio 50:1.
§ Incubated with SBA without separation of SBA^+ and SBA^- cells.
‖ Not done.
¶ Incubated with SBA followed by removal of agglutinated, SBA^+ cells by sedimentation on 2.5% BSA.

of responder cells with anti-NK-1.2 sera + RC before MLC reduced NK and the MLC induced AK drastically, whereas allospecific killing was actually somewhat enhanced (Fig. 2).

Thus, suppression of the early, anomalous killing did not alter the subsequent allospecific response significantly. Conversely, it was possible to induce normal levels of AK in populations with impaired potential for allospecific killing. Splenocytes from mice treated with adult thymectomy, irradiation, and reconstitution with fetal liver cells were able to generate early YAC-1 killing in MLC, whereas the allospecific response to RBL-5 was virtually abolished (Table VIII). Responder cells from normal mice depleted of Thy-1.2-positive cells by the monoclonal reagent at a dilution of 10 μg/ml + RC also yielded normal AK levels and suppressed allospecific activity (Table VIII).

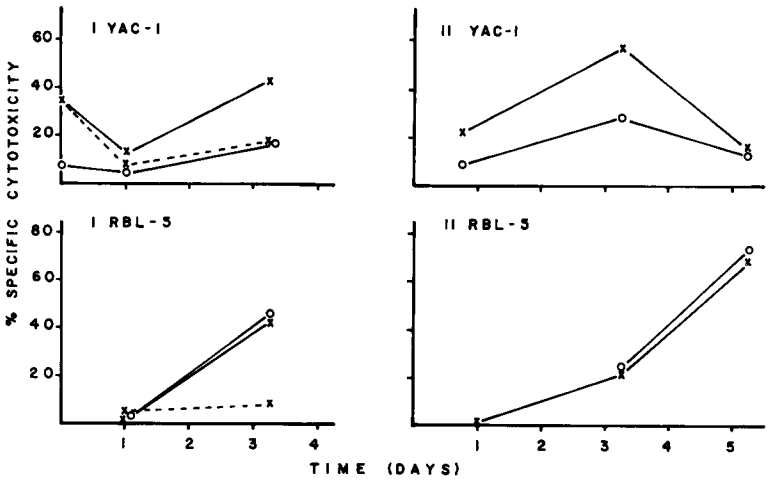

FIG. 2. Cytotoxic responses of (CBA × A)F_1 spleen cells treated with anti-NK 1.2 antiserum before MLC. The diagrams show percent lysis with responder cells treated with FCS (1:30) + RC (—×—) or antiserum (1:30) + RC (○) before allostimulation with C57B1 cells, and control cells treated with FCS (1:30) + RC and then cultured without allogeneic stimulator cells (--×--). The anti-NK 1.2 antiserum was CE anti-CBA in experiment I and (CE × NZB)F_1 anti-CBA in experiment II. Effector/target ratio was 50:1.

TABLE VIII

AK Response of (CBA × A)F_1 Cells with Depressed Allospecific CTL Potential

Experiment	Responder cells and treatment*	Stimulator cells	Percent lysis‡			
			YAC-1		RBL-5	
			80 h	120 h	80 h	120 h
1	(CBA × A)F_1 control (× FL)	C57Bl	47	28	38	49
	(CBA × A)F_1 thymectomy (AT × FL)	C57Bl	44	31	10	10
2	(CBA × A)F_1-FCS 1/50 + RC	None	15	—	2	—
		C57Bl	29	8	30	80
	(CBA × A)F_1 anti Thy 1.2 (10 μg/ml) + RC	None	11	—	1	5
		C57Bl	46	38	3	23

* All responder and stimulator were spleen cells. See Materials and Methods for detailed protocols on the various treatments of animals and cells.

‡ Effector/target ratio was 100:1 in experiment 1 and 50:1 in experiment II.

These results obtained with anti Thy-1.2 pretreatment of responders were somewhat surprising, since the AK effectors themselves were always sensitive (70% reduction) to the same concentration of anti-Thy-1.2 antibody (Table IV). The results therefore imply that the AK-precursor is a nonthymus-processed cell that increases Thy-1 expression during activation in culture. However, we could not conclude that induction of AK is completely independent of thymus-processed cells. Many T cell functions, such as production of T cell growth factor and IFN upon mitogenic or antigenic stimulation, are radioresistant (27, 28). Our thymectomy protocol did not exclude the presence of some remaining, radioresistant T cells, and all our MLC also included T cells among the irradiated splenic stimulator cells. To critically assess any possible involvement of T cells, we therefore used congenitally athymic nude mice as the

source of responder as well as stimulator cells. Indeed, when C57B1 *nu/nu* spleen cells were used as stimulators, AK could be induced only with BALB/c +/*nu*, and not BALB/c *nu/nu* responders (Table VIII). Similar results were obtained with stimulator spleen cells from thymectomized and reconstituted C57B1 mice and responder cells from Swiss +/*nu* and *nu/nu* littermates. The AK of allostimulated cells as well as the rather high YAC-1 killing induced with Swiss +/*nu* cells cultured alone did not occur when Swiss *nu/nu* were used as responders (Table IX). The AK response was partially restored by addition of irradiated +/*nu* (but not by *nu/nu*) cells to the Swiss *nu/nu* responders (Table IX).

Comparisons of Anomalous Killers and NK_c Cells. It has previously been reported (4) that cytotoxicity mediated by non-T cells may arise spontaneously in FCS-containing cultures. These were defined by their ability to lyse WEHI-164 fibrosarcoma cells very efficiently, and were termed "NK_c-cells" (11). Fig. 3 shows a comparison of cytotoxic responses against YAC-1 and WEHI-164 in the same control and allostimulated control cultures of (CBA × A)F_1 spleen cells. Efficient anomalous killing against YAC-1 required allostimulation, and peaked by day 3 or 4. In contrast, NK_c cells against WEHI-164 were induced as efficiently in the control as in the allostimulated cultures, and showed peak cytotoxicity around day 5 - 6 (as previously reported (4, 11). Furthermore, while the YAC-1 killing could be markedly reduced by anti-Thy-1.2. + RC treatment, the WEHI-164 killing remained intact. These results indicate that NK_c and AK cells represent functionally distinct populations.

Discussion

In a previous study (10), we have described AK cells, Thy-1^+ cytotoxic cells, which are activated early in (CBA × A)F_1 → C57B1_x MLC and lyse semisyngeneic and xenogeneic tumor targets in the same preferential order as NK-cells. The results of the experiments presented here provided a further insight in this phenomenon,

TABLE IX
Induction of AK Requires Thymus-processed Cells

Experiment	Responder cells	Anomalous YAC-1 killing after 80 hrs of culture			
		Without stimulator cells		C57Bl *nu/nu* stimulator cells	
		50:1*	25:1	50:1	25:1
1	BALB/c +/*nu*	3	1	*20*	*14*
	BALB/c *nu/nu*	2	0	0	0
				C57Bl AT × FL stimulator cells‡	
2	Swiss +/*nu*	20	15	37	36
	Swiss *nu/nu*	1	2	9	6
	Swiss *nu/nu* + Swiss nu/nu_x§	0	0	6	1
	Swiss *nu/nu* + Swiss +/nu_x	7	7	20	15

* Effector/target ratio.
‡ See Materials and Methods for details on thymectomy protocol.
§ 20 × 10^6 irradiated (1,000 rad) *nu/nu* or +/*nu* cells added to the culture.

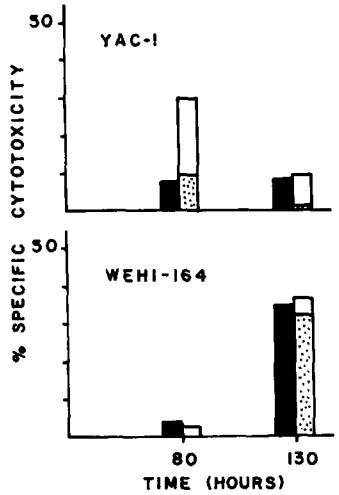

FIG. 3. Comparison of cytotoxic responses to YAC-1 and WEH-164 in control and allostimulated spleen cell cultures. Effector cells from (CBA x A)F_1 (■) and (CBA × A)F_1 → C57B1$_x$ cultures (□) were tested in a 5 h ^{51}Cr-release assay at an effector/target ratio of 100:1. MLC-derived effectors were also tested after treatment with monoclonal anti-Thy-1.2 ascites (10 μg/ml) + RC (▤), except against WEHI-164 at 80 h. Cells treated with complement alone (not shown) did not differ significantly in cytotoxic potential from untreated cells.

especially with regard to the cellular requirements and mechanisms of activation for anomalous killing.

Induction of AK depended upon cells that express NK 1.2 and bind SBA. Several different inbred mouse strains and F_1 hybrid crosses could serve as donors for AK responsive cells, which were derived from a nonadherent radiosensitive spleen cell population. Lymph node cells responded poorly, whereas spleen depleted of cells expressing a high density of Thy-1.2 antigen as well as spleen from AT × FL animals responded like the appropriate controls. With the exception of radiosensitivity, all these characteristics fit one operationally defined population, namely NK cells (28). It is therefore conceivable that the only cells required among the responders are closely related, if not identical to NK cells (phenotype: NK 1^+. Thy-$1^{-/weak}$), and that these are the direct precursors of AK cells (phenotype: NK-$1^{-/weak}$. Thy-1^+).

Furthermore, induction of AK required immune stimulation. When responder cells were incubated in autologous serum alone in the absence of any known antigens, no anomalous killing was registered. Incubation in FCS-containing medium usually induced at least weak cytotoxicity against YAC-1 cells. The addition of irradiated allogeneic spleen cells to the cultures reproducibly resulted in stronger anti YAC-1 cytotoxicity, regardless of whether FCS or NMS was used. Thus, antigen stimulation was required in our system, although it may be that mitogen-induced stimulation of the immune system could work as well. Similar to what has been reported for antigen and mitogen-induced T cell activation (25, 30), adherent cells were required for the induction of AK in allogeneic MLC. We do not know whether the cytotoxicity generated in cultures containing FCS (but no stimulator cells) represents the same general phenomenon as alloantigen-induced anomalous killing, but it is probably also dependent on antigenic stimulation. It is of course possible that FCS contains molecules that trigger heterologous lymphocytes by binding to true mitogen receptors

(i.e., polyclonally distributed, nonantigen-specific receptors). However, it is not necessary to invoke this postulate as the FCS does contain bovine specific proteins that mouse lymphocytes can recognize as antigenic (31).

Our results with spleen cells from *nu/nu* mice demonstrated that the confrontation of precursors of AK cells with antigen in the presence of adherent cells is not sufficient to induce AK. Mature thymus-processed cells are required as well, although the data indicate that these are at least partly radioresistant, and therefore do not necessarily have to be included in the responder population. It is important to remember that allogeneic MLC are bidirectional, and even if proliferation of stimulator cells is prevented by irradiation, they may still respond by secretion of interleukins and IFN (27, 28). The antigen required for induction of AK may therefore even be provided by the cytotoxic precursors themselves, or other cells in the responder population, which are recognized by the stimulator cells. Especially in MLC, in which responders were depleted of allospecific CTL potential, and in the F_1 anti-parental MLC, this may be the major type of antigen recognition taking place in the cultures.

We therefore suggest that the triggering reaction in our system is antigen recognition by T cells, which subsequently activate NK cells (which may not have to see antigen at all), as suggested for a similar murine system by Pacucci et al. (32, 33) and by Herberman et al. (34) for a human system. Our interpretation that NK cells are direct precursors of AK cells is enforced by the observation that the two have a very similar recognition pattern. This was shown most clearly by the results with IFN-treated YAC-1 cells and the YAC-1 variants, all of which are recognized less well by NK cells, although they have a higher or at least as high H-2 expression as YAC-1 cells (17, 21). We have previously shown that K562 cells, which do not express any detectable HL-A products at all, are sensitive to murine as well as human AK (5, 10), suggesting that their recognition is not based on interaction with the MHC products on the target cells. However, we do not imply that all autologous cytotoxicity arising in culture is independent of H-2 and mediated by effectors derived from NK-cells. It has previously been shown that culturing of murine spleen cells with specific growth factors or with certain batches of FCS leads to generation of at least two killer cell populations (35), which must be different from AK cells. The NK_c-cells (11), probably analogous to NK_M-cells (35), seem to be derived from a splenic natural effector cell directed primarily against solid tumor targets, the so-called NC cell (36). NK_c-cells lyse WEHI-164 fibrosarcoma targets and were shown here to differ from AK by several important criteria (Fig. 2). A second type of killer cell arising in culture without allostimulation expresses Ly-2 apart from Thy-1 and kills NK-insensitive targets such as P815. As emphasized previously by other investigators (11, 35), such cytotoxic effectors share all characteristics of CTL, and the term NK cells for these may cause confusion. Such CTL may be recognizing self-MHC, as described in other systems (37). Specific, antiparental cytotoxicity induced in certain F_1-antiparental MLC may also fit in this category of anti-self-MHC reactions (38). However, the specific F_1-antiparental effectors show some peculiar characteristics in comparison with allospecific CTL; they cannot be induced with spleen cells from mice younger than 3 wk, nor is it possible to "prime" them with preimmunization (38). They may therefore be derived from NK-cells, which share these properties, rather than from T cells. If F_1-antiparental effectors are NK cells, they should lyse YAC-1, and indeed, Kumar et al. (39) have previously reported that YAC-1 cytotoxicity is generated in

addition to the activity against parental EL-4 cells in (DBA × C57B1)F1 → C57B1 MLC, and we observed a similar generation of YAC-1 killers in other F_1-antiparental MLC combinations in the present study. Our results further indicate that failure to demonstrate NK 1.2 antigen on cultured effectors is not sufficient evidence to conclude that they are conventional CTL rather than Thy-1^+ NK cells. The origin of F_1-antiparental effectors in various systems must therefore be studied with fractionation of responder cells before MLC.

The two effector cells induced in allogeneic MLC (anomalous and allospecific) are clearly distinguishable at the precursor level, and we conclude that murine anomalous killing is not just an additional function of conventional specific CTL, as has been suggested for culture induced NK-like killers in some human systems (16). Our data are more compatible with the reports of Pacucci et al. (32, 33), showing activation of non-T killer cells against autologous solid tumors dependent on allostimulated T cells, and of Chun et al. (40), showing activation of NK cells through Con A-stimulated T cells. However, our findings differ from these two reports in two respects: AK were found to be derived from radiosensitive precursors and to be clearly sensitive to anti Thy-1.2 treatment. In the two other systems, NK activation was registered even after all input cells had been irradiated and with the effector cells being resistant or only marginally sensitive to anti-Thy-1. One factor that may account for these differences is that we used a response against one allogeneic donor, which although it may involve a high frequency of T cells (41), is still quite weak in comparison with stimulation by Con A (40) or a pool of different allogeneic donors (33). Thus, our simple allogeneic stimulus would favor activation of NK cells by a proliferation-dependent process with concurrent modulation of their surface markers to weaker expression of NK-1 and stronger expression of Thy-1. We have no definite proof that AK cells are dividing. However, they are blasts as judged by their distribution in velocity sedimentation (10) and in discontinuous BSA density gradients, (unpublished observation). It is possible to induce and maintain proliferation of NK-like cells in vitro by cultivation in Con A supernatants (42) as well as in purified IL-2 (35, 42). Interestingly, cells in such NK lines all express Thy-1 antigen, in contrast to fresh splenic NK cells (42). One may therefore ask whether these lines really represent NK cells and not conventional T cells. However, they seem to have the same quantitative distribution of certain surface markers as NK cells (42), and we show here that similar cells can be observed in MLC only if responder cells contain NK activity, indicating that they are derived from NK cells. We therefore suggest that some of the different subsets or maturation stages that account for surface marker heterogeneity in the fresh NK population (22–24) do not survive, or alternatively are pushed further in differentiation upon the influence of signals released during an immune response (or maintenance in Con A supernatant). This would explain the shift in surface markers between the fresh NK population on one hand, and AK cells and NK lines on the other hand. A phenotype switch has also been observed for human NK cells cultured in conditioned media (OKM-1^+ OKT-3^- → OKM-1^- OKT-3^+; J. Ortaldo, personal communication). Our observation that the AK effector cells seem to be more sensitive to anti-Thy-1 treatment than their precursors supports the suggestion of Minato et al. (35) that Thy-1^- cells are precursors of Thy-1^+ cells within a putative NK lineage. The same authors concluded that only NK cells of the Thy-1^+ phenotype respond to IL-2, and Henney et al. (43) demonstrated that IL-2 and IFN act synergistically in

NK activation. Because IFN activates NK cells in a proliferation-independent process (44), and because IFN is known to inhibit cell division rather than to stimulate it, it is likely that induction of AK does involve other signals released in MLC, such as IL-2.

In our previous report (10) we pointed at the similarities between anomalous killers and certain cytotoxic responses elicited in vivo (45, 46). This parallel to in vivo phenomena is now even more apparent, since it has recently been shown that intraperitoneal injection of allogeneic cells induce Thy-1^+ NK cells in the peritoneal exudate (47). This occurred in H-2 identical but Mls-incompatible combinations (similar to that we observed in vitro), as well as with single H-2K, D, or I locus differences. Moreover, it has been demonstrated that "activated" NK-cells from mice infected with lymphocyte choriomeningitis virus (LCMV) are also larger and more sensitive to anti-Thy-1.2 treatment than native NK-cells (48). Although the augmentation of NK activity appeared in parallel with increased splenic interferon levels already 2–3 d after LCMV infection, the NK-cells with high expression of Thy-1 were not detected until 5–6 d after infection, in the period preceeding the peak of the specific CTL response against LCMV (48). This suggests that other (or additional) factors than IFN may be required for the development of the Thy-1^+ NK-phenotype in vivo.

In conclusion, the surface markers of NK cells and their descendents AK cells indicate that they are more closely related to T cells than to B cells, suggesting that the events determining commitment to the B as opposed to T (and possibly other) lineages occur earlier than acquirement of NK function in the ontogeny. This does not necessarily mean that NK cells are precursors in the T lineage, and that they mature into CTL via the anomalous killer cell stage. In the system described here, the two latter functions certainly occurred independently.

Summary

Anomalous killer cells are Thy-1^+ blasts that are cytolytic to the natural killer (NK)-sensitive lymphoma YAC-1, and that can be detected early (day 3–4) in the period preceeding the allospecific cytotoxic T lymphocyte (CTL) response in (CBA × A)F1 → C57B1 mixed leukocyte culture (MLC). We have investigated the origin and nature of anomalous killing (AK), with special emphasis on its relation to NK- and allospecific CTL-activity. AK was shown to be distinct from the previously described "NK_c-cells" induced by cultivation in fetal calf serum (FCS)-supplemented medium when these two reactivities were examined in parallel. AK was detected in either FCS- or normal mouse serum (NMS)-supplemented allogeneic MLC, indicating that the response was not dependent on mitogenic or antigenic properties of heterologous serum. In addition to several H-2-incompatible combinations, AK was also observed in an Mls-incompatible (but H-2 compatible) and two F_1-antiparental MLC responder/stimulator combinations. AK cells showed a similar selectivity pattern to NK cells, as demonstrated in cold target inhibition and direct cytotoxicity assays using variant or interferon-modulated YAC-1 cells with low expression of NK target structures. The AK-cells were NK-$1.2^{-/weak}$. Thy-1.2^+, although they seem to be derived from non-adherent radiosensitive cells which are closely related, if not identical, to NK-cells (NK-1.2^+. Thy-$1.2^{-/weak}$, as they could not be readily induced in responder populations with low NK-activity but normal allospecific CTL potential.

Conversely, an in vivo thymectomy protocol or treatment of normal spleen cells with monoclonal anti-Thy-1.2 + C reduced the allospecific CTL response drastically but did not affect the AK response. Anomalous killers were not observed when MLC were prepared with responder as well as stimulator cells devoid of mature T cells. In such a combination, the AK response could be partially restored by the addition of irradiated +/*nu* (but not *nu/nu*) responder cells to the cultures. When normal (nonnude) spleen cells were used as responders, induction of AK did not require the presence of T cells in the stimulator population, whereas the removal of adherent and phagocytic cells from stimulators abrogated the response.

Taken together, the results suggest that AK represents activation, blast transformation, and surface marker modulation of NK cells induced by alloantigen-stimulated T cells, resulting in Thy-1$^+$ cytolytic cells with similar properties to those described for NK lines. Although AK cells may be regarded as a more T cell-like NK phenotype, their induction is neither necessary, nor sufficient for generation of specific CTL in MLC.

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References

1. Shustik, C., I. R. Cohen, R. S. Schweitz, E. Latham-Griffin, and S. D. Waksal. 1976. T-lymphocytes with promiscuous cytotoxicity. *Nature (Lond.)*. **263:**699.
2. Peck, A. B., L. C. Andersson, and H. Wigzell. 1977. Secondary *in vitro* response of T-lymphocytes to non-H-2 alloantigens. Self-H-2-restricted responses induced in heterologous serum are not dependent on primary-stimulating non-H-2-alloantigens. *J. Exp. Med.* **145:**802.
3. Zielske, J. V., and S. H. Golub. 1976. Fetal calf serum-induced blastogenic and cytotoxic responses of human lymphocytes. *Cancer Res.* **36:**3842.
4. Burton, R. C., S. E. Chism, and N. L. W. Warner. 1977. *In vitro* induction of tumor specific immunity. VII. Does autosensitisation occur with *in vitro* culture of T-lymphocytes? *J. Immunol.* **119:**1329.
5. Seeley, J. K., and S. A. Golub. 1978. Studies on cytotoxicity generated in human mixed lymphocyte cultures. I. Time course and target spectrum of several distinct concomitant cytotoxic activities. *J. Immunol.* **120:**1415.
6. Masucci, M. G., E. Klein, and S. Argov. 1980. Disappearance of the NK effect after explantation of lymphocytes and generation of similar nonspecific cytotoxicity correlated to the level of blastogenesis in activated cultures. *J. Immunol.* **124:**2458.
7. Jondal, M., and S. Targan. 1978. In vitro induction of cytotoxic effector cells with spontaneous killer cell specificity. *J. Exp. Med.* **147:**1621.
8. Callewaert, D. M., J. J. Lightbody, J. Kaplan, J. Jaroszewski, W. D. Peterson, and J. C. Rosenburg. 1978. Cytotoxicity of human peripheral lymphocytes in cell mediated lympholysis, antibody-dependent cell-mediated lympholysis and natural cytotoxicity assays after mixed lymphocyte culture. *J. Immunol.* **122:**81.
9. Bolhuis, R. L. H., and M. Shellekens. 1981. Induction of natural killer cell activity and allocytotoxicity in human mixed peripheral blood lymphocyte culture. *Scand. J. Immunol.* **13:**401.
10. Kärre, K., and J. K. Seeley. 1979. Cytotoxic Thy 1.2-positive blasts with NK-like target selectivity in murine mixed lymphocyte cultures. *J. Immunol.* **123:**1511.

11. Burton, R. 1980. Alloantisera selectively reactive with NK cells: characterization and use in defining NK cell classes. *In* Natural Cell-mediated Immunity against Tumors. R. B. Herberman, editor. Academic Press, Inc., New York. 19–36.
12. Chun, M., V. Pasanen, U. Hämmerling, G. F. Hämmerling, and M. K. Hoffmann. 1979. Tumor necrosis serum induces a serologically distinct population of NK cells. *J. Exp. Med.* **150:**426.
13. Roder, J. C. 1980. Phenotypic characteristics of NK-cells in the mouse. *In* Natural Cell-mediated Immunity against Tumors. R. B. Herberman, editor. Academic Press, Inc., New York. 161–172.
14. Gidlund, M., O. Haller, A. Örn, E. Ojo, P. Stern, and H. Wigzell. 1980. Characteristics of murine NK-cells in relation to T-lymphocytes and K-cells. *In* Natural Cell-mediated Immunity against Tumors. R. B. Herberman, editor. Academic Press, Inc., New York. 79–90.
15. Kaplan, J., and D. Calleweart. 1978. Expression of human T-lymphocyte antigens by natural killers. *J. Natl. Cancer Inst.* **60:**961.
16. Klein, E. 1980. Natural and activated cytotoxic T-lymphocytes. *Immunol. Today* **I:**IV.
17. Grönberg, A., R. Kiessling, E. Eriksson, and M. Hansson. 1981. Variants from a MLV-induced lymphoma selected for decreased sensitivity to NK-lysis. *J. Immunol.* **127:**1734.
18. Click, R. E., L. Benck, and B. J. Alter. 1972. Immune responses *in vitro* I. Culture conditions for antibody synthesis. *Cell. Immunol.* **3:**264.
19. Burton, R. C., and and H. J. Winn. 1981. Studies on natural killer (NK) cells. 1. NK cell specific antibodies in CE anti-CBA serum. *J. Immunol.* **26:**1985.
20. Lake, P., E. A. Clark, M. Khorshidi, and G. Sunshine. 1979. Production and characterization of cytotoxic Thy-1 antibody secreting hybrid cell lines. Detection of T cell subsets. *Eur. J. Immunol.* **9:**875.
21. Welsh, R. M., K. Kärre, M. Hansson, L. A. Kunkel, and R. W. Kiessling. 1981. Interferon mediated protection of normal and tumor target cells against lysis by mouse natural killer cells. *J. Immunol.* **126:**219.
22. Mattes, M. J., S. O. Sharrow, R. B. Herberman, and H. T. Holden. 1979. Identification and separation of Thy-1 positive mouse spleen cells active in natural cytotoxicity and antibody dependent cell-mediated cytotoxicity. *J. Immunol.* **123:**1851.
23. Pollack, S. B., M. R. Tam, R. C. Nowinski, and S. L. Emmons. 1979. Presence of T cell-associated surface antigens on murine NK cells. *J. Immunol.* **123:**1818.
24. Koo, G. C., J. B. Jacobsson, C. J. Hämmerling, and K. Hämmerling. 1980. Antigenic profile of murine natural killer cells. *J. Immunol.* **125:**1003.
25. Wagner, H., M. Feldmann, W. Boyle, and J. W. Schrader. 1972. Cell mediated immune response *in vitro.* III. The requirement for macrophages in cytotoxic reactions against cell-bound and subcellular allo antigens. *J. Exp. Med.* **136:**331.
26. Reisner, Y., M. Biniaminov, E. Rosenthal, N. Sharon, and B. Ramot. 1976. Use of soy bean agglutinin for the separation of mouse T- and B-lymphocytes. *Biochem. Biophys. Res. Commun.* **72:**1585.
27. Wagner, H., and M. Röllinghoff. 1978. T-T Cell interactions during *in vitro* cytotoxic allograft responses. I. Soluble products from activated Ly-1^+ T cells trigger autonomously antigen primed Ly-23^+ T cells to cell proliferation and cytolytic activity. *J. Exp. Med.* **148:**1523.
28. Kirschner, H., R. Zawatzky, and V. Schirrmacher. 1979. Interferon production in murine mixed lymphocyte culture. I. Interferon production caused by differences in the H-2K and H-2D region but not by differences in the I region or the M locus. *Eur. J. Immunol.* **9:**97.
29. Kiessling, R., and H. Wigzell. 1979. An analysis of the murine NK cell as to structure, function and biological relevance. *Immunol. Rev.* **44:**165.

30. Habu, S., And M. C. Raff. 1977. Accessory cell dependence of lectin induced proliferation of mouse T-lymphocytes. *Eur. J. Immunol.* **7:**451.
31. Forni, G., and I. Green. 1976. Heterologous sera: a target for *in vitro* cell mediated cytotoxicity. *J. Immunol.* **116:**1561.
32. Paciucci, P. A., S. MacPhail, J. M. Zarling, and F. H. Bach. 1980. Lysis of syngeneic solid tumor cells by alloantigen stimulated mouse T and non-T cells. *J. Immunol.* **124:**370.
33. Paciucci, P. A., S. MacPhail, F. M. Bach, and J. M. Zarling. 1980. Requirements for T-cells in alloantigen-induced generation of non-T cell-mediated cytotoxicity against syngeneic mouse sarcoma cells. *J. Immunol.* **125:**36.
34. Herberman, R. B., J. Y. Djeu, H. D. Kay, J. R. Ortaldo, C. Riccardi, G. D. Bonnard, H. T. Holden, R. Fagnani, A. Santoni, and P. Puccetti. 1979. Natural killer cells: characteristics and regulation of activity. *Immunol. Rev.* **44:**43.
35. Minato, N., L. Reid, and B. J. Bloom. 1981. On with heterogeneity of natural killer cells. *J. Exp. Med.* **154:**750.
36. Stutman, O. P., R. Dien, G. Wisun, G. Pecoraro, and E. C. Lattime. 1980. Natural cytotoxic (NC) cells against solid tumors in mice: some target cell characteristics and blocking of cytotoxicity by D-mannose. *In* Natural Cell-mediated Immunity against Tumors. R. B. Herberman, editor. Academic Press, Inc., New York. 949–962.
37. Altman, A., and D. H. Katz. 1980. The induction of autoreactive T-lymphocytes by allogeneic effect factor (AEF). Relevance to normal pathways of lymphocyte differentiation. *Immunol. Rev.* **51:**3.
38. Nakano, K., I. Nakamura, and G. Cudkowicz. 1981. Generation of F_1-hybrid cytotoxic T-lymphocytes specific for self H-2. *Nature (Lond.).* **289:**559.
39. Kumar, V., E. Luevano, and M. Bennett. 1979. Hybrid resistance to EL-4 lymphoma cells. 1. Characterization of natural killer cells that lyse EL-4 cells and their distinction from marrow-dependent natural killer cells. *J. Exp. Med.* **150:**531.
40. Chun, M., G. Fernandes, and M. K. Hoffmann. 1981. Mechanism of NK cell activation: relationship between $Qa5^+$ NK cells and lymphocytes. *J. Immunol.* **126:**331.
41. Ryser, J. E., and H. R. MacDonald. 1979. Limiting dilution analysis of alloantigen-reactive T-lymphocytes. I. Comparison of precursor frequencies for proliferative and cytotoxic responses. *J. Immunol.* **122:**1691.
42. Dennert G., G. Yogeeswaran, and S. Yamagata. 1981. Cloned cell lines with natural killer activity. Specificity, function, and cell surface markers. *J. Exp. Med.* **153:**545.
43. Henney, C. S., K. Kuribayashi, D. E. Kern, and S, Sillis. 1981. Interleukin-2 augments natural killer activity. *Nature (Lond.).* **291:**335.
44. Senik, A., B. B. Kolb, A. Örn, and M. Gidlund. 1981. Study of the mechanism for *in vitro* activation of mouse NK-cells by interferon. *Scand. J. Immunol.* **12:**51.
45. Welsh, R. M. 1978. Cytotoxic cells induced during lymphocytic choriomeningitis virus infection of mice. I. Characterization of natural killer cell induction. *J. Exp. Med.* **148:**163.
46. Blanden, R. V., and I. D. Gardner. 1976. The cell-mediated immune response to ectromelia virus infection. I. Kinetics and characteristics of the primary effector T-cell response *in vitro*. *Cell. Immunol.* **22:**271.
47. Clark, E. A., and R. D. Holly. 1981. Activation of natural killer (NK) cells *in vivo* with H-2 and non-H-2 alloantigens. *Immunogenetics.* **12:**221.
48. Kiessling, R., E. Eriksson, L. Hallenbeck, and R. Welsh. 1980. A comparative analysis of the cell surface properties of activated versus non-activated mouse natural (NK) cells. *J. Immunol.* **125:**1551.