

## CLOINED CELL LINES WITH NATURAL KILLER ACTIVITY

### Specificity, Function, and Cell Surface Markers\*

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Recent advances in tissue culture procedures have resulted in the *in vitro* establishment of permanent lymphoid cell lines, which retain their physiological functions and antigen-recognition specificity (1, 2). So far, the cell lines under study are all derived from thymus-dependent lymphocytes which display various functions, e.g., cytolytic activity (1-3), helper activity (4, 5), and delayed type hypersensitivity.<sup>1</sup> The availability of such cell lines and their clonal derivatives facilitates a more thorough study of the functions, receptor specificities, and effector molecules of T cells. Another cell type, whose nature and function are rather poorly understood within the immune system, is the natural killer (NK)<sup>2</sup> cell (6, 7). NK cells do not have immunological memory and are present at high levels without prior priming. They may therefore represent an important first defense against newly arising neoplastic cells. The literature has suggested that, although NK cells recognize a large variety of apparently unrelated targets, there may be distinct cell surface antigens with which NK effector cells interact. This was hypothesized from experiments that used unlabeled competitor cells to inhibit target cell lysis (8, 9). Furthermore, it has been reported that the binding of NK effector cells to their targets can be inhibited by a putative isolated target structure (8, 10). For instance, target structures isolated from human cells, although they inhibit NK effector binding to human NK targets, do not inhibit effector binding to mouse cell targets and vice versa. It was concluded from these experiments that NK cells have specific receptors which enable them to distinguish between mouse and human target antigens. Because of the many unresolved questions about NK cells, we established permanent lines of NK cells *in vitro* (11, 12). Here we report the results of experiments in which we have cloned one of these lines and determined its target specificity, cytolytic activity, and cell surface markers.

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<sup>1</sup> Weiss, S., and G. Dennert. T cell lines active in the delayed type hypersensitivity reaction. Submitted for publication.

<sup>2</sup> *Abbreviations used in this paper:* ADCC, antibody-dependent cell-mediated cytotoxicity; asialo G<sub>M1</sub>, ceramide-glucose-galactose-*N*-acetyl-galactosamine-galactose; asialo G<sub>M2</sub>, ceramide-glucose-galactose-*N*-acetyl-galactosamine; a/t, attacker to target cell ratio; CD, ceramide-glucose-galactose; CM, ceramide-glucose; Con A, concanavalin A; Con A CM, medium conditioned by culturing spleen cells in the presence of concanavalin A; DMEM, Dulbecco's modified Eagle's minimal essential medium; FCS, fetal calf serum; FMF, flow microfluorometry; G<sub>D1a</sub>, ceramide-glucose-galactose (sialic acid) *N*-acetyl galactosamine-galactose (sialic acid); G<sub>D1b</sub>, ceramide-glucose-galactose (sialic acid-sialic acid) *N*-acetyl galactosamine-galactose; G<sub>M1</sub>, ceramide-glucose-galactose (sialic acid) *N*-acetyl galactosamine-galactose; G<sub>M2</sub>, ceramide-glucose-galactose (sialic acid) *N*-acetyl galactosamine; G<sub>M3</sub>, ceramide-glucose-galactose-sialic acid; GSL, glycosphingolipids; NK, natural killer.

### Materials and Methods

*Animals.* BALB/c mice were bred at The Salk Institute, San Diego, Calif., and BALB/c nu/nu mice were purchased from Sprague Dawley (Madison, Wis.).

*Tumor Cell Lines.* The C57Bl/6 thymic lymphoma EL4 (H-2<sup>b</sup>) and the C58 thymic lymphoma R1.G1 (H-2<sup>k</sup>) cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum. The BALB/c cell line B/C-N (H-2<sup>d</sup>) and its relatively NK-sensitive derivative B/C-N 10ME HD A5R1 (B10ME), were kindly provided by Drs. P. Patek, J. Collins, and M. Cohn (The Salk Institute) and grown in DMEM containing 10% fetal calf serum (FCS). The human cell line, Chang liver, was also grown in the above medium. The A-strain mouse cell line, YAC-1 (H-2<sup>a</sup>), and its NK lysis-resistant derivative YAC-8 (kindly provided by Drs. R. Kiessling and M. Hansson, Karolinska Institute, Stockholm, Sweden), the human cell lines Molt 4 and K562, and the BALB/c mouse cell line RL $\delta$ 1 (kindly provided by R. B. Herberman, National Institutes of Health, Bethesda, Md.) were grown in RPMI-1640 containing 10% FCS.

*Preparation of Conditioned Medium.* Mouse spleen cells were harvested and erythrocytes were lysed by treatment with distilled water for 10 s, and a concentrated NaCl solution was added to bring the salt concentration back to 0.15 M. After washing in Hanks' balanced salt solution, spleen cells ( $1-2 \times 10^7$ /ml) were cultured for 48 h in RPMI-1640 complete tissue culture medium in the presence of 10  $\mu$ g/ml concanavalin A (Con A). The cell supernate was collected by centrifugation and filtered through a 0.45- $\mu$ m Millipore filter (Millipore Corp., Bedford, Mass.; 13).

*Cell-mediated Cytotoxicity.* Cytotoxic activity was assayed on <sup>51</sup>Cr-labeled target cells in RPMI-1640 medium containing 10% FCS as described previously (11). The following equation was used to express cytotoxicity: % cytotoxicity = [(experimental <sup>51</sup>Cr release - spontaneous <sup>51</sup>Cr release)/(maximal <sup>51</sup>Cr release - spontaneous <sup>51</sup>Cr release)]  $\times$  100. Maximal release was determined by freezing and thawing the target cells three times. The mean values of duplicate samples and the standard errors of the means (SEM) were calculated. Tumor cells were the targets for the NK assay, and both tumor cells and chicken erythrocytes were targets for the antibody-dependent cell-mediated cytotoxicity (ADCC) assay.

*Determination of Cell Surface Markers.* Cell surface marker determinations with antibody were performed by flow microfluorometry (FMF). Briefly,  $1-3 \times 10^6$  cells in 0.2 ml phosphate-buffered saline containing 0.2% serum albumin and 0.25% Na Azide were reacted for 30 min at 4°C with the first antibody, and were then washed and incubated with fluorescein-conjugated rabbit anti-mouse or anti-rat immunoglobulin serum (14). After washing, the cells were suspended at  $2-5 \times 10^6$ /ml for analysis using a fluorescence-activated cell analyzer. T24/31.7 and I3/2.3 are monoclonal rat anti-mouse antibodies specific for Thy-1 and T200, respectively (14, 15). Monoclonal rat anti-mouse Lyt-1 and Lyt-2 reagents were derived from 53-7.313 and 53-6.72 hybridoma cell lines kindly provided by Drs. J. A. Ledbetter and L. A. Herzenberg (16).

For analysis of glycosphingolipids,  $4-5 \times 10^6$  cells were labeled with 2  $\mu$ Ci/ml ([1-<sup>14</sup>C]-galactose, 60.5 mCi/mM; New England Nuclear Corp., Boston, Mass.) in 5 ml medium conditioned by culturing spleen cells in the presence of Con A (Con A CM) for 20-24 h. The labeled cells were washed and extracted with a 2:1 chloroform/methanol dilution, and neutral glycosphingolipids (GSL) were purified and analyzed by thin-layer chromatography, followed by autoradiography as described before (17). Ganglioside fractions were purified and analyzed with carrier human brain ganglioside as described before (17). All GSL were identified by chromatographic mobilities with standards of known structure. Neutral GSL standard mixtures were obtained by acid hydrolysis and purification of human brain ganglioside mixtures. These mixtures were comprised of ceramide-glucose (CM), ceramide-glucose-galactose (CD); ceramide-glucose-galactose-N-acetyl-galactosamine-galactose (G<sub>M1</sub>), and ceramide-glucose-galactose-N-acetyl-galactosamine (G<sub>M2</sub>). Standard ganglioside mixtures consisted of dog erythrocyte (ceramide-glucose-galactose-sialic acid [G<sub>M3</sub>]), Tay-Sachs brain G<sub>M2</sub>, adult human brain G<sub>M1</sub>, ceramide-glucose-galactose (sialic acid) N-acetyl galactosamine-galactose (sialic acid) (G<sub>D1a</sub>), ceramide-glucose-galactose (sialic acid-sialic acid) N-acetyl galactosamine-galactose (G<sub>D1b</sub>), and G<sub>T</sub>. Thin-layer plates were developed with orcinol reagent to detect neutral glycolipids and resorcinol reagent for staining of gangliosides as described before (17).

## Results

*Cloning and Specificity of NK Cell Lines.* We previously reported that spleen cells of normal or nu/nu BALB/c mice cultured in tissue-culture Con A CM maintain cytolytic activity characteristic for NK cells (11, 12). Interestingly, cytotoxicity increased with time, suggesting that NK effector cells may have proliferated, whereas other cells disappeared. Several cultures were set up from BALB/c nu/nu and normal spleen cells and supplemented on a weekly basis with Con A CM. When assayed on YAC-1 targets, they showed variable cytolytic activity that could reach 80% lysis in a 6-h assay at an attacker to target cell (a/t) ratio of 3:1. By comparison with fresh BALB/c spleen cells, this would amount to at least a 20-fold increase in activity on a per cell basis. A panel of NK-sensitive and -resistant targets was used to examine the cytolytic specificity of several cell cultures. Fig. 1 is a representative experiment. The individual cultures show relatively high cytolytic activity on YAC-1 targets but much lower activity on the NK-resistant YAC-8. Similarly, B10ME is more efficiently lysed than B/CN, from which it was derived as a NK-sensitive target. The three human targets, Molt 4, K562, and Chang liver, show intermediate lysis, whereas the two mouse thymic lymphomas, EL4 and R1.G1, are resistant to NK lysis at these a/t ratios. Two important points emerged from these results and from other experiments not shown here. First, the relative sensitivity of different targets to lysis is constant and independent of the NK cell line or the fresh spleen cell preparation used. Second, the absolute level of cytotoxicity caused by a particular effector line, and therefore the relative ranking of individual NK lines on a given target, varies from experiment to experiment. This variability is presumably a reflection of the particular growth condition of the line at the time of assay.

To explore whether recognition structures for mouse and human targets are expressed clonally, NK 11 was cloned by limiting dilution at one cell per well. Five isolates were grown and assayed on YAC-1, Molt 4, K562, and EL4 (Fig. 2). Results show that there is no difference between the lytic specificity of these clones and that of normal spleen cells. Therefore, if there are specific receptors on NK 11 effectors, they are not clonally distributed.

*NK Cell Lines Do Not Show Antibody-dependent Cell-mediated Lysis.* The failure to detect distinct target specificities by cloning NK cell lines could suggest that NK cells do not express specific receptors. This, however, would leave open the possibility that NK cells can acquire target specificity by interaction with the Fc portion of target-specific antibody. It has been reported that NK and ADCC effector cells specific for nucleated targets may be identical (18–20). Comparison of spleen effector cells and the NK 11 cell line for their ability to lyse chicken erythrocytes in the presence of antitarget cell antibody demonstrated that NK 11 is unable to effect ADCC of erythroid targets (data not shown). Next, the ability of NK 11 to lyse EL4 targets was tested in the presence of anti-H-2<sup>b</sup> alloantisera. Whereas normal spleen cells showed significant antibody-dependent cell-mediated lysis on EL4 (Table I, experiment 1), no lysis of EL4 was seen with NK 11. Similar results were obtained with two other targets, RL $\delta$ 1 and Chang liver, both of which are susceptible to lysis in an ADCC reaction with normal spleen cells. Cell line NK 11 therefore appears to be an ADCC-negative NK effector cell.

*Lytic Activity of NK Cell Lines in the Presence of Con A.* The observation that NK 11 does not lyse targets in an ADCC reaction could be due either to the absence of Fc

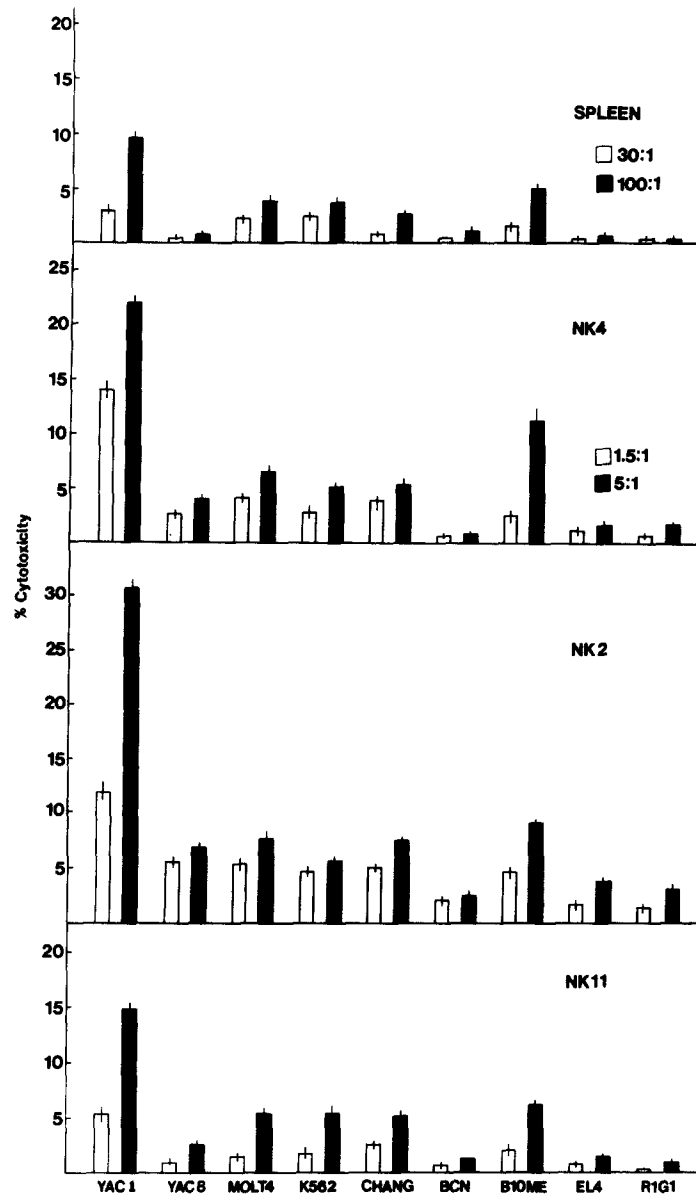


FIG. 1. Cytolytic specificity of three NK cell lines. NK 2 and NK 4 were derived from BALB/c nu/nu spleen cells and NK 11 from a normal BALB/c spleen. A/t ratios were 30:1 and 100:1 for spleen cells, and 1.5:1 and 5:1 for the NK cell lines. The 5-h time point is given.

receptors on these NK cells or to their failure to lyse targets after binding. To analyze whether the binding of NK effectors to targets invariably results in target lysis, Con A was used to agglutinate NK 11 cells with various targets in the cytotoxicity assay. Results showed (Fig. 3) that the cytolytic activity of NK-sensitive targets such as YAC-1 and RL31 can be dramatically enhanced by the addition of 4  $\mu\text{g}/\text{ml}$  Con A. Lysis of the relatively NK-resistant target YAC-8, which shows variable lysis with NK

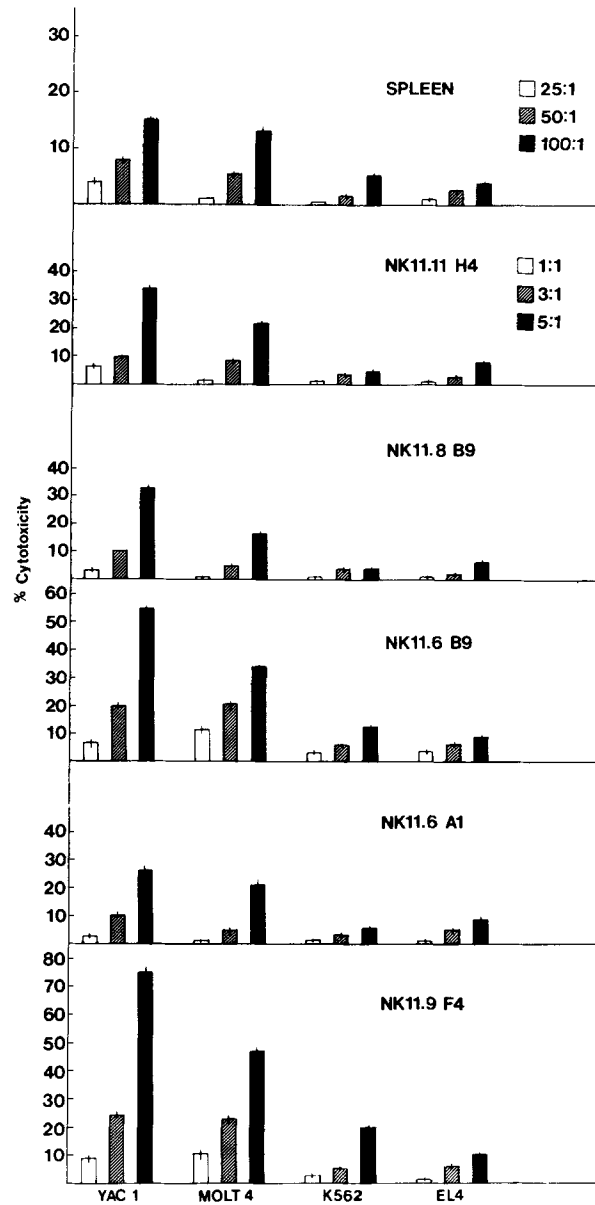


FIG. 2. Cytolytic specificity of cloned NK sublines. Sublines were cloned at one cell per well and five isolates were grown and assayed for cytotoxicity at ratios of 1:1, 3:1, and 5:1 in a 7-h assay. Spleen effector cells were used at ratios of 25:1, 50:1, and 100:1.

lines (Fig. 1), is also enhanced, but cytotoxicity in the presence of lectin does not approach those levels obtained with YAC-1 and RL $\delta$ 1. Cell line R1.G1, which is a good target for T killer cells (11) but is poorly lysed on NK cell lines (Fig. 1), shows only a small increase in cytotoxicity in the presence of Con A. This result suggests that the amount of cytotoxicity caused by NK effectors might be a function of two variables. One is the efficacy of binding of the NK cell to its target. Apparently, even targets that are lysed

TABLE I  
Failure of NK-11 to Effect ADCC and Autokilling

	Effector cells	a/t	Serum dilution*	Target	Cytotoxicity‡	
					4 h	7 h
					%	
Experiment 1	Spleen§	100:1	1 1:100	EL4	8 ± 0.7	15 ± 0.1
	Spleen	100:1	1 1:300	EL4	9 ± 0.4	22 ± 0.2
	Spleen	100:1	1 1:1,000	EL4	7 ± 0.3	20 ± 1.7
	Spleen	100:1	1 1:3,000	EL4	3 ± 0.04	13 ± 1.8
	Spleen	100:1	2 1:100	EL4	16 ± 1.5	35 ± 0.6
	Spleen	100:1	2 1:300	EL4	15 ± 0.6	24 ± 0.2
	Spleen	100:1	2 1:1,000	EL4	9 ± 1.2	25 ± 2.9
	Spleen	100:1	2 1:3,000	EL4	5 ± 0.5	15 ± 0.12
	NK-11-3	6:1	— —	YAC-1	41 ± 0.3	73 ± 1.5
	NK-11-3	6:1	1 1:100	EL4	<1	<1
	NK-11-3	6:1	1 1:300	EL4	<1	<1
	NK-11-3	6:1	1 1:1,000	EL4	<1	<1
	NK-11-3	6:1	1 1:3,000	EL4	<1	<1
	NK-11-3	6:1	2 1:100	EL4	<1	<1
	NK-11-3	6:1	2 1:300	EL4	3 ± 1.44	<1
	NK-11-3	6:1	2 1:1,000	EL4	1 ± 0.84	1 ± 0.96
NK-11-3	6:1	2 1:3,000	EL4	1 ± 7	<1	
Experiment 2	NK-11-6-D6	20:1	— —	YAC-1	—	97 ± 1.8
	NK-11-6-D6	5:1	— —	YAC-1	—	91 ± 0.6
	NK-11-6-D6	1:1	— —	YAC-1	—	69 ± 0.6
	NK-11-6-D6	20:1	— —	NK-11-6-D6	—	<1
	NK-11-6-D6	5:1	— —	NK-11-6-D6	—	<1
	NK-11-6-D6	1:1	— —	NK-11-6-D6	—	<1

\* Serum 1 B10 D2 × A/J α EL4. Serum 2 B10 BR × AKR/J α EL4.

‡ Spleen cells together with targets in the absence of antibody served as controls.

§ C3H spleen cells.

well, such as YAC-1 and RL $\delta$ 1, are not optimally lysed, due to a lack of optimal binding that can be augmented by the addition of Con A. The second variable is the sensitivity of the target to the lytic events that occur after binding. Thus, YAC-8 and especially R1.G1 targets appear to be relatively resistant to these lytic events.

*Cell Surface Antigens of NK Effector Cells.* The cell surface antigenic phenotype of NK cells is still a somewhat controversial subject (20, 21–23). It was therefore of interest to analyze the cell surface antigens of our NK cell lines. This was done by using monoclonal antibody and FMF. Results (data not shown) revealed the presence of Thy-1 and T200 antigen in quantities comparable to those on our previously described allospecific T killer cell line, C.C3.11.75 (3). In contrast to this T killer line, there was no detectable Lyt-1 antigen on NK 11 cells, nor was there any Lyt-2 antigen present.

Because of the many reports dealing with unique GSL on NK cells (24–26), NK 11 was labeled with [<sup>14</sup>C]galactose and subjected to GSL analysis. Lipids were extracted and separated by thin-layer chromatography. The plates were incubated on film for autoradiography and subsequently stained with orcinol. In Fig. 4 (A–C), one of two independent experiments is presented, in which NK 11 and two allospecific T cell lines, C.C3.11.75 (3) and B6.C.7.76 (27), are compared. The autoradiographs of the neutral glycolipids (Fig. 4A) show significant amounts of asialo G<sub>M1</sub> and asialo G<sub>M2</sub> on NK 11. In contrast, the two allospecific T cell lines, C.C3.11.75 and B6.C.7.76, show no asialo G<sub>M2</sub>. Interestingly, C.C3.11.75, which is a cytolytic T cell line (3), displays asialo G<sub>M1</sub>, whereas B6.C.7.76, which is not cytolytic (27), does not. Appar-

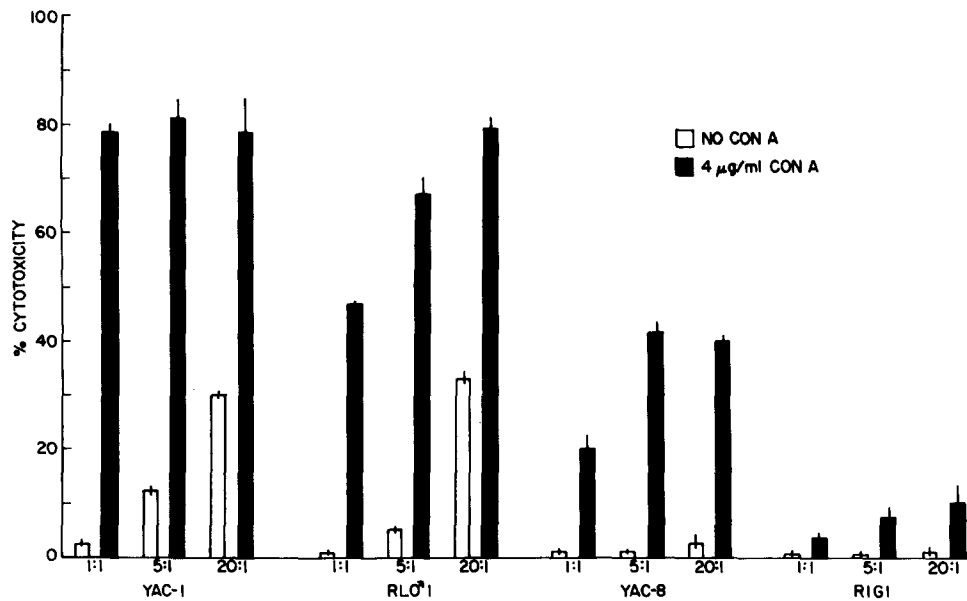


FIG. 3. Ability of NK 11.6.D6 to lyse YAC-1, YAC-8, RLO-1, and RIG1 in the absence or presence of 4 µg/ml Con A. Spontaneous  $^{51}\text{Cr}$  release from targets was determined in the presence of 4 µg/ml Con A, although Con A did not increase spontaneous  $^{51}\text{Cr}$  release. The 4-h time point is given at the a/t ratios indicated.

ently, the amount of asialo  $\text{G}_{\text{M}1}$  on C.C3.11.75 is small compared with that on NK effectors, because both asialo  $\text{G}_{\text{M}1}$  and asialo  $\text{G}_{\text{M}2}$  can be detected on NK 11 by orcinol staining. They cannot be seen by staining on both allospecific T cell lines (Fig. 4B). The ganglioside patterns of NK 11 and the allospecific T cell lines are shown in Fig. 4C. There was some variability in the patterns among C.C3.11.75, B6.C.7.76, and NK 11 in the components migrating in the areas corresponding to chromatographic mobilities of standard  $\text{G}_{\text{M}2}$  and  $\text{G}_{\text{M}3}$ . The demonstration of well-labeled gangliosides in both experiments served as a control for proper labeling of the cell lines examined. Identical experimental results were obtained with cloned subline NK 11.8 (data not shown).

The most remarkable feature of the neutral glycolipid profile of NK 11 is that it possesses high levels of asialo  $\text{G}_{\text{M}1}$  and asialo  $\text{G}_{\text{M}2}$  as compared with allospecific T cell lines. Asialo  $\text{G}_{\text{M}2}$  has not been reported for NK effectors but has been observed with some regularity on NK-sensitive targets (28).<sup>3</sup> Because of the presence of asialo  $\text{G}_{\text{M}2}$  on NK targets, we examined whether NK effector cells are able to lyse each other. Table I, experiment 2, shows that NK 11-6 lyses YAC-1 targets efficiently, but is incapable of lysing NK 11-6 targets. The mere presence of asialo  $\text{G}_{\text{M}2}$  on target cells is therefore not sufficient for lysis to occur.

### Discussion

Cloning NK cell lines enabled us to study whether NK effectors possess antigen receptors and whether these receptors are clonally distributed. Results clearly showed that target specificities do not segregate upon cloning. This finding is compatible with

<sup>3</sup> Yogeeswaran, G., and R. Kiessling. Manuscript submitted for publication.

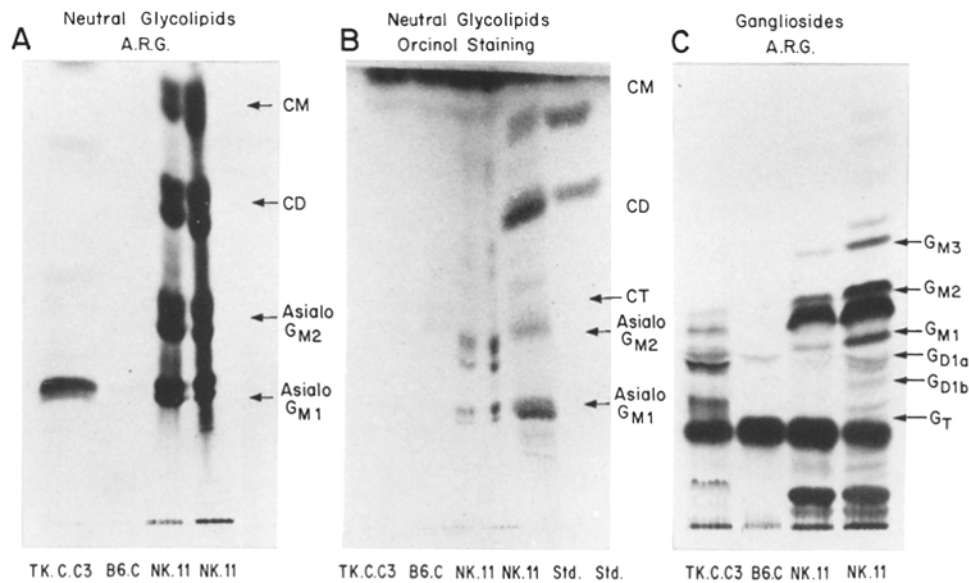


FIG. 4. Neutral glycolipids and gangliosides of line NK 11 and the allospecific T cell lines C.C.3.11.75 (TK C.C.3) and B6.C.7.75 (B6.C). Thin layer chromatograms show radiochemically (A) and chemically (B) detectable neutral glycosphingolipids and radiochemically detectable gangliosides (C). GSL fractions corresponding to amounts to  $4-5 \times 10^6$  cells ( $3.5-80 \times 10^3$  cpm) were separated on silica gel-gypsum thin layer plates and developed either in chloroform:methanol:water (65:30:8 by volume, resulting lower phase) for neutral GSL or chloroform:methanol:water:ammonia (60:35:7:1 by volume) for gangliosides. For autoradiography plates were exposed to Kodak No Screen NS54T film for 10-24 d.

three hypotheses. First, the NK effector does not possess antigen receptors; second, it possesses receptors that recognize identical antigenic specificities on the targets used in these experiments; and third, it expresses a multitude of receptors, each with a different specificity. If NK effectors have no receptors, then target cells that cannot be lysed by NK effector cells would have to be resistant to lysis because failure to lyse targets could not be due to improper binding. If NK effectors possess receptors, targets would either have to lack target antigens or be lysis resistant.

Our experiments clearly show that targets such as YAC-8 and especially R1.G1 are relatively lysis resistant to NK lysis, even in the presence of Con A. This result is compatible with NK cells possessing or lacking antigen receptors. However, the case for antigen-specific receptors on NK cells is substantially weakened by the finding that in the presence of Con A even a sensitive target like YAC-1 is substantially better lysed than in its absence. Thus, it appears that NK cells either do not have receptors or have ones with low affinity for the target. It is possible that attachment of NK cells to various targets may occur in different degrees, depending on the cell surface structures of the two cells, even if no specific receptors as such exist. This hypothesis could account for the observation that in cold target inhibition assays, good targets tend to inhibit well, whereas bad targets do not (8-10, but see 29). Therefore, cold target inhibition assays do not conclusively prove the presence of specific receptors on NK effectors.

The failure to uncover any evidence for specific receptors on our *in vitro* cultured NK cells does not rule out the possibility that, *in vivo*, these cells act specifically with



the help of target-specific antibody. Results showed that neither erythroid nor lymphoid targets are lysed in an ADCC reaction. This finding is at variance with results suggesting that NK effectors may express ADCC activity (18, 19). It is possible to explain this discrepancy with the hypothesis that there is some heterogeneity in the NK effector-cell population. This hypothesis would be in agreement with cell surface marker analysis of NK cells. Although our NK cell lines express significant amounts of Thy-1 antigen, in agreement with previous reports (19, 20, 22, 23), there is also evidence for NK activity in Thy-1<sup>-</sup> cell populations (20). Hence, it is possible that distinct Thy-1<sup>+</sup>, as well as Thy-1<sup>-</sup>, NK effectors exist. It is by no means excluded that the Thy-1<sup>-</sup> and Thy-1<sup>+</sup> NK effector cells previously described (20) belong to one cell population containing cells with different degrees of maturity and, consequently, with different amounts of Thy-1 cell surface antigen. Thus, it is still unknown whether NK activity represents activities expressed by different cell types still to be characterized.

The presence of Thy-1 antigen on NK cells raises the question of the relationship of NK effector cells and T killer cells. We have shown that NK 11 is Thy-1<sup>+</sup>, T200<sup>+</sup>, and Lyl 1<sup>-</sup>2<sup>-</sup>, although two of our allospecific T cell lines, one of which is cytolytic, are Thy-1<sup>+</sup>, T200<sup>+</sup>, and Lyl 1<sup>+</sup>2<sup>-</sup>. The lack of Lyl antigens may therefore be characteristic for NK effectors. Another important characteristic of NK cells may be the expression of the neutral glycolipids asialo G<sub>M1</sub> and asialo G<sub>M2</sub>. Both glycolipids are expressed in amounts that make detection by both orcinol staining and metabolic labeling possible. Asialo G<sub>M1</sub>, however, can also be detected by metabolic labeling on the cytolytic allospecific T cell line C.C3.11.75. This agrees with previous reports in which T killer cells could be lysed by anti-asialo G<sub>M1</sub> antibody in the presence of complement (22). The significance of the observation that asialo G<sub>M1</sub> is detectable on the cytolytic T cell line but not on the line that is not cytolytic, cannot be evaluated until more cell lines of this sort are analyzed. The presence of asialo G<sub>M2</sub> on NK effectors has not been reported yet and poses an interesting question because this neutral glycolipid has been noticed with some regularity on NK-sensitive targets (28). The possibility that asialo G<sub>M2</sub> plays a role in NK effector target interaction was not supported by our experiments because NK effectors do not show mutual cytolysis. Therefore, at present, there is no strong evidence for an NK receptor, nor is there any indication of what the target structure, if any, on NK targets might be. Furthermore, the antigenic profile of our NK cell lines, although it distinguishes them from functional T cells, does not allow us to assign these cells to a particular hematopoietic cell lineage. Because of their expression of Thy-1 and presence in nu/nu mice, they may belong to prethymic T cells.

Although questions pertaining to NK cells, such as the presence of receptors and mechanism of cytolysis, remain unresolved, they can obviously be much better analyzed with cloned NK cell lines now available. In addition, easier exploration of the *in vivo* effects of NK cells on tumor growth and F<sub>1</sub> hybrid resistance to parental marrow grafts is now possible. An important consideration for such studies will be the stability of NK lines. In this regard, it is well known that T killer lines growing in Con A CM, in contrast to T killer lines which grow in response to antigenic stimulation, may undergo considerable chromosomal variation and loss of function (30). Since NK lines have been in culture for a comparatively short time, no statement about their stability can be made. It has been noticed, however, that some cloned sublines

show decreased cytolytic activities over a period of some months, although others do not. Sporadic recloning of sublines may therefore be necessary.

### Summary

Cell lines with natural killer (NK) activity grown from naive spleen cells cultured in medium conditioned by spleen cells proliferating in the presence of concanavalin A (Con A) were characterized. One NK cell line was cloned and assayed on several human and mouse NK-sensitive targets to analyze whether target specificities segregate upon cloning. Results showed that NK clones display target specificities identical to NK cells in normal spleen. This suggests that NK cells have no clonally distributed specific receptors to a given target. They may, however, have receptors which recognize identical antigens on all NK-sensitive targets or may possess multiple receptors for different target specificities. NK lines could not be demonstrated to possess activity in antibody-dependent cell-mediated cytotoxicity, nor did they effect mutual lysis. In the presence of Con A, NK cells exhibited dramatically enhanced lysis of NK-sensitive targets but only a slight increase in lysis of NK-insensitive targets. This indicates that the degree of lysis of an NK target is a function of two variables: effector binding to the target and target sensitivity to lysis. Furthermore, it suggests that the affinity of the putative antigen receptors on NK effectors must be rather weak. Cell surface marker analysis reveals that NK cell lines are Thy 1.2<sup>+</sup>, Lyt-1<sup>-</sup>2<sup>-</sup>, T200<sup>+</sup>, asialo GM<sub>1</sub><sup>+</sup>, and asialo GM<sub>2</sub><sup>+</sup>. These markers distinguish NK cells from cytolytic thymus-derived lymphocytes, without resolving the question of classification within a given hematopoietic cell lineage.

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### References

1. Dennert, G., and M. DeRose. 1976. Continuously proliferating T killer cells specific for H-2<sup>b</sup> targets: selection and characterization, *J. Immunol.* **116**:1601.
2. Gillis, S., and K. A. Smith. 1977. Long term culture of tumor specific cytotoxic T cells. *Nature (Lond.)* **268**:154.
3. Dennert, G. 1979. Cytolytic and proliferative activity of a permanent T killer cell line. *Nature (Lond.)* **277**:476.
4. Glasebrook, A. L., and F. W. Fitch. 1980. Alloreactive cloned T cell lines. *J. Exp. Med.* **151**: 876.
5. Augustin, A. A., and A. Coutinho. 1980. Specific T helper cells that activate B cells polyclonally. *J. Exp. Med.* **151**:587.
6. Roder, J. C., and R. Kiessling. 1978. Target-effector interaction in the natural killer cell system. *Scand. J. Immunol.* **8**:135.
7. Herberman, R. B., and H. T. Holden. 1978. Natural cell-mediated immunity. *Adv. Cancer Res.* **27**:305.
8. Roder, J. C., A. Rosen, E. M. Fenyő, and F. A. Troy. 1979. Target-effector interaction in the natural killer cell system: isolation of target structures. *Proc. Natl. Acad. Sci. U. S. A.* **76**: 1405.

9. Koide, Y., and M. Takasugi. 1978. Specificities in natural cell-mediated cytotoxicity against lymphoblastoid cell lines. I. Selective inhibition by cross competition. *Eur. J. Immunol.* **8**: 818.
10. Roder, J. C., L. Ahrlund Richter, and M. Jondal. 1979. Target-effector interaction in the human and murine natural killer system. *J. Exp. Med.* **150**:471.
11. Dennert, G., and R. Hyman. 1980. Functional Thy-1<sup>+</sup> cells in cultures of spleen cells from nu/nu mice. *Eur. J. Immunol.* **10**:583.
12. Dennert, G. 1980. Cloned lines of natural killer cells. *Nature (Lond.)*. **287**:47.
13. Rosenberg, S. A., P. J. Spiess, and S. Schwarz. 1978. In vitro growth of murine T cells. I. Production of factors necessary for T cell growth. *J. Immunol.* **121**:1946.
14. Dennert, G., R. Hyman, J. Lesley, and I. S. Trowbridge. 1980. Effects of cytotoxic monoclonal antibody specific for T200 glycoprotein on functional lymphoid cell populations. *Cell. Immunol.* **53**:350.
15. Trowbridge, I. S. 1978. Interspecies spleen-myeloma hybrid producing monoclonal antibodies against mouse lymphocyte surface glycoprotein, T200. *J. Exp. Med.* **148**:313.
16. Ledbetter, J. A., and L. A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* **47**:362.
17. Yogeeswaran, G., and B. S. Stein. 1980. Glycolipids of metastatic variant RNA virus transformed non-producer Balb/3T3 cell lines: altered metabolism and cell surface exposure. *J. Natl. Cancer Inst.* **65**:967.
18. Ojo, E., and H. Wigzell. 1978. Natural killer cells may be the only cells in normal mouse lymphoid cell populations endowed with cytolytic ability for antibody-coated tumor target cells. *Scand. J. Immunol.* **7**:297.
19. Herberman, R. B., J. Y. Djeu, H. D. Kay, J. R. Ortaldo, C. Riccardi, G. D. Bonnard, H. T. Holden, R. Fagnani, A. Santori, and P. Puccett. 1979. Natural killer cells: characteristics and regulation of activity. *Immunol. Rev.* **44**:43.
20. 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**:2851.
21. Kasai, J., J. C. Leclerc, F. W. Shen, and H. Cantor. 1979. Identification of Ly 5 on the surface of 'natural killer' cells in normal and athymic inbred mouse strains. *Immunogenetics.* **8**:153.
22. Gidlund, M., O. Haller, A. Orn, E. Ojo, P. Stern, and H. Wigzell. 1980. In *Natural Cell-mediated Immunity to Tumors*. R. B. Herberman, editor. Academic Press, Inc., New York. 79.
23. Koo, G. C., J. B. Jacobson, G. J. Hämmerling, and U. Hämmerling. 1980. Antigenic profile of murine natural killer cells. *J. Immunol.* **125**:1003.
24. Schwarting, G. A., and A. Summers. 1980. Gangliotetraosylceramide is a T cell differentiation antigen associated with natural cell-mediated cytotoxicity. *J. Immunol.* **124**:1691.
25. Young, W. W., S. I. Hakomori, J. M. Durdik, and C. S. Henney. 1980. Identification of ganglio-N-tetraosylceramide as a new cell surface marker for murine natural killer (NK) cells. *J. Immunol.* **124**:199.
26. Kasai, M., M. Iwamori, Y. Nagai, K. Okumura, and T. Tada. 1980. A glycolipid on the surface of mouse natural killer cells. *Eur. J. Immunol.* **10**:175.
27. Dennert, G., S. L. Swain, J. D. Waterfield, J. F. Warner, and R. W. Dutton. 1980. Fine specificity of two allospecific T cell lines: recognition of private specificities in the H-2 IA subregion. *Eur. J. Immunol.* In press.
28. Young, W. W., J. Durolik, D. Undal, S. Hakomori, and C. Henney. 1980. Lymphoma cell glycolipids and NK susceptibility. *J. Immunol.* In press.

29. Collins, J. C., P. Q. Patek, and M. Cohn. 1981. Tumorigenicity and lysis by natural killers. *J. Exp. Med.* **153**:89.
30. Nabholz, M., A. Conzelman, A. Oreste, M. North, W. Haas, P. Pohlit, H. von Boehmer, H. Hengartner, J. P. Mach, H. Engern, and J. P. Johnson. 1980. Established murine cytolytic T-cell lines as tools for a somatic cell genetic analysis of T cell functions. *Immunol. Rev.* **51**: 125.