MULTIPLE ACTIVITIES OF A CLONED CELL LINE MEDIATING NATURAL KILLER CELL FUNCTION*

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Many immunologic cells require exposure to antigen to display detectable levels of specific function. One cell type requires no known previous exposure to antigen; however, it displays a specificity for particular target cells (1–5). These cells lyse certain neoplastic or normal cells without deliberate immunization and have thus been termed natural killer (NK)[1] cells. Because these cells in the mouse comprise <5% of spleen cells, they have been defined according to their expression of cell surface antigens and their ability to lyse certain tumor target cells. NK cell activity is eliminated by complement-dependent lysis with anti-Ly-5, anti-NK-1, anti-Qat-4, and anti-Qat-5, and is resistant to anti-Lyt antisera (5–7). Activity can also be eliminated after multiple treatment with anti-Thy-1 antisera and complement (8). Two patterns of NK reactivity have been found in mouse spleen cells. In both cases, spleen cells lyse virus-infected tumor targets such as the YAC-1, RLδ, MBL-2, or RBL-5 lymphomas. After activation with bacille Calmette-Guérin (9), interferon (IF) inducers (10–12), certain tumors (13), or undefined factors (14, 15), NK cells acquire the ability to lyse additional tumors such as the EL4 lymphoma or P815 mastocytoma, which are relatively resistant to nonactivated NK cells.

The relation of NK cells to the cells that mediate antibody-dependent cellular cytotoxicity is unclear. Although some data support the hypothesis that NK cells can mediate both kinds of activities (16, 17), it has not been possible to settle these questions because homogeneous populations of NK cells have been difficult to isolate.

Just as myelomas have facilitated the analysis of B cells and immunoglobulin, cloned populations of cells showing NK activity can better define the cellular and molecular basis of the activities described above. Several continuous cell lines have been described recently that lyse the YAC-1 lymphoma (18, 19). We have cloned a cell population from $Ig^{-}Ly\text{-}5^{+}$ spleen cells that lyses the YAC-1 lymphoma 100 times more efficiently than NK-containing spleen populations. Unlike other lines described previously, these cells also lyse the EL4 lymphoma and P815 mastocytoma, and mediate another specialized function: antibody-dependent cellular cytoxicity (ADCC).

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[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; CE, clonal efficiency; CCM, complete conditioned medium; CM, conditioned medium; CTL, cytotoxic T lymphocytes; FCS, fetal calf serum; IF, interferon; LPS, lipopolysaccharide; mc, monoclonal; NK, natural killer; PAS, periodic acid-Schiff peroxidase; SRBC, sheep erythrocytes.

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Materials and Methods

Animals. All T cells for cloning were obtained from C57BL/6 mice. Cells from C57BL/6 or BALB/c mice (obtained from The Jackson Laboratory, Bar Harbor, Maine) were used to produce conditioned medium (CM; see below).

Antisera. Lyt-1.2 and Lyt-2.2 antisera, prepared as described (20), were kindly donated by Dr. F. W. Shen; NK-1 antisera by Dr. G. Koo; monoclonal (mc) anti-Thy-1.2 by Dr. Ed Clark; mc-anti-Lyt-1 and mc-anti-Lyt-2 by Drs. J. Ledbetter and L. Herzenberg; and mc-anti-Qat-5 by Dr. U. Hammerling. Expression of Ly-1 and Ly-2 by each clone was determined with both Lyt alloantisera and mc Lyt antibodies by immunofluorescence.

Cell Culture and Cloning. Culture conditions for initiation and maintenance of cell lines have been described (21). Briefly, cells were diluted into microwells (3040; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) at estimated final concentrations of 100, 10, 1, and <1 cells/well. Each well contained 0.1 ml of complete conditioned medium (CCM; 21), and irradiated (1,500 rad) cell monolayers from different tissues (final concentrations 4–8 × 10^5 cells/well). Cultures were supplemented every 2 d with 30 μl CCM until colonies were visible (10 d–3 wk) in wells that initially received <100 cells. Cloning efficiency (CE) was calculated by Poisson distribution (22). At least 96 wells were used to calculate each CE. The fraction of negative wells was plotted as a function of the logarithm of initial cell number. Single cells from colonies arising at frequencies <36% with CE >5% were regrown twice after single cell micromanipulation. With some exceptions, the characteristics of these initial cell colonies were stable and identical to those of cloned populations derived from them by single cell micromanipulation. Separate tests of more than 12 different cell colonies showed that 3–10 daughter clones derived from each expressed the cell surface antigens, morphology, and function of the parent colony. After the initial cloning procedure, cells were expanded in cultures containing CM in the absence of irradiated cell monolayers (21). Doubling times ranged from 24 to 48 h for all cells tested. Cells could be expanded to numbers >10^8. At least 20 different cloned populations have been propagated for 8 mo in vitro.

Frozen Cells for Storage. Cloned cells were frozen in liquid N_2 and thawed with complete recovery of growth and function, by the use of a modification (21) of the method of Lionetti and colleagues (23).

Positive Selection of Ly-5$^+$ Cells. Cells were prepared according to a previous method (7). Briefly, spleen cells from B6 mice were passed through a nylon-wool column and treated with anti-Thy-1.2 (final dilution 1:20) and a selected rabbit serum as a source of complement. These cells were washed three times and incubated for 30 min at 4°C with anti-Ly-5.1 (final dilution of 1:20) in the absence of complement. 5 ml of these cells (10^7/ml) were added to petri dishes (100 × 20 mm, Falcon 3003) coated with a monolayer of sheep erythrocytes (SRBC) conjugated to protein A. Coupling of SRBC to protein A was performed by the method of Parrish and Hayward (24); protein A-SRBC monolayers were prepared by the method of Stulting and Burke (25).

After a 15-min incubation of lymphocytes at 4°C, the plates were gently swirled and incubated an additional 15 min at 4°C. Unbound (nonadherent) cells were removed by gently aspirating the supernatant with a Pasteur pipette. Bound cells were released from the monolayers after the addition of 2 ml of distilled water for 15 s, followed by two additions of 2 ml of phosphate-buffered saline. This procedure lyses SRBC and permits recovery of the bound lymphocyte population. The recovered cell population (unbound plus bound) represents ≧80 of the starting (input) cell population.

NK Assay. Lysis of the YAC-1 lymphoma, an NK-sensitive tumor (2, 26; >35% specific lysis at an effector:target ratio of 1:1) and lack of lysis against the radiation-induced C57BL RL-12 lymphoma, an NK-resistant tumor, was used to define NK activity. Briefly, 2 × 10^4 ^{51}Cr-labeled target cells (YAC-1, RL-12, EL-4, or P815) were incubated in minimum essential medium plus 10% fetal calf serum (FCS) alone or with various cloned cells for 3.5 h at 37°C in a humidified atmosphere with 5% CO_2 (5). The amount of gamma radioactivity released from triplicate cultures was measured. Cytoxicity is expressed as percent specific lysis according to the formula:

$$\text{Percent lysis} = \frac{\text{experimental release-spontaneous release}}{\text{maximal release (HCl)-spontaneous release}} \times 100.$$

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Preparation of Lipopolysaccharide (LPS)-activated B Cells. To obtain purified B cells, spleen cells were suspended in anti-Lyt-1.2 plus anti-Lyt-2.2 plus mc-anti-Thy-1.2 at optimal lytic concentrations for 30 min at room temperature, washed, and incubated in a selected rabbit serum (complement) at 37°C for 40 min. To ensure depletion of all T cells, this treatment was followed by further incubation of the remaining cells with mc-anti-Thy-1.2 (final dilution, 1:1,000) and rabbit complement at 37°C for 30 min. These remaining cells did not secrete appreciable Ig after 4 d of culture in vitro unless purified T cells were added.

These cells were incubated with 20 μg/ml *Escherichia coli* 055:B5 LPS (Sigma Chemical Co., St. Louis, Mo.) at 37°C in a humified atmosphere with 10% CO_2 in modified Dulbecco's modified minimum essential medium, 4% FCS, 5×10^{-5} M 2-mecaptoethanol and glutamine (2 mM). Cells (2×10^6/ml) were incubated for 72 h before radiolabeling with ^{51}Cr for use in the cytotoxic assay.

Results

Isolation and Cloning of NK Cells. Ly-5$^+$ cells were enriched ~12-fold from spleen cell populations of B6 mice (7) before cells were distributed into microwells for cloning. The frequency of functionally active NK colonies arising from this starting population was 27% compared with a frequency of 1% arising from unselected spleen cell populations. 12 clones expressed the Thy-1$^+$Lyt-1$^-$2$^-$Ly-5$^+$Qat4$^+$5$^+$ cell surface components. One clone, containing cells expressing this surface phenotype (Cl.Ly-1$^-$2$^-$NK-1$^+$/11), was examined in detail. Subsequent analysis showed that it expressed the NK-1 antigen, too. Lytic activity of these cells against the YAC-1 lymphoma was ~100-fold more potent than NK cells in spleen populations (Table I; Fig. 1).

Histochemistry. Wright-Giemsa (27) periodic-acid-Schiff (PAS) peroxidase (28) and nonspecific esterase (29) reactions were performed by described protocols (Fig. 2). Cl.Ly-1$^-$2$^-$NK-1$^+$/11 did not contain peroxidase, nonspecific esterase, or PAS-positive

TABLE I

Comparison of Functional CE of NK Cells after Positive Selection

Source of cells for cloning	CE	NK activity (% positive)	CE of NK cells
			%
A. Normal mouse serum plus rabbit complement spleen	4.5	26.7	1.2
B. Ig$^-$Thy-1$^-$Ly-5$^+$ spleen cells	67	40	26.8

Spleen cells were (A) sera treated or (B) enriched 12-fold for NK activity (7). CE were computed using the Poisson distribution.

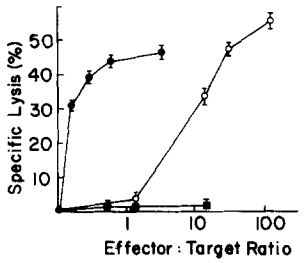

FIG. 1. Cell-mediated cytotoxicity against the YAC-1 lymphoma. Uncloned spleen cells (○), Cl.Ly-1$^-$2$^-$NK-1$^+$/11 (●) and Cl.Ly-1$^-$2$^-$NK-1$^-$/1 (■) were incubated with radiolabeled YAC-1 lymphoma. No cells lysed the NK-resistant RL-12 leukemia.

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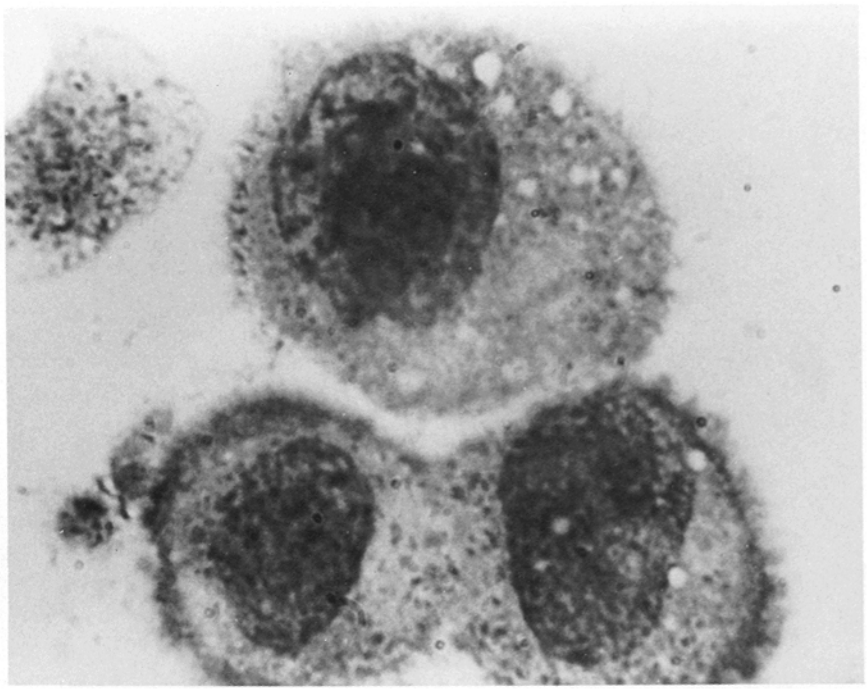

FIG. 2. Wright-Giemsa stain of Cl.Ly-1$^-$2$^-$NK-1$^+$/11. Cytocentrifuged × 100.

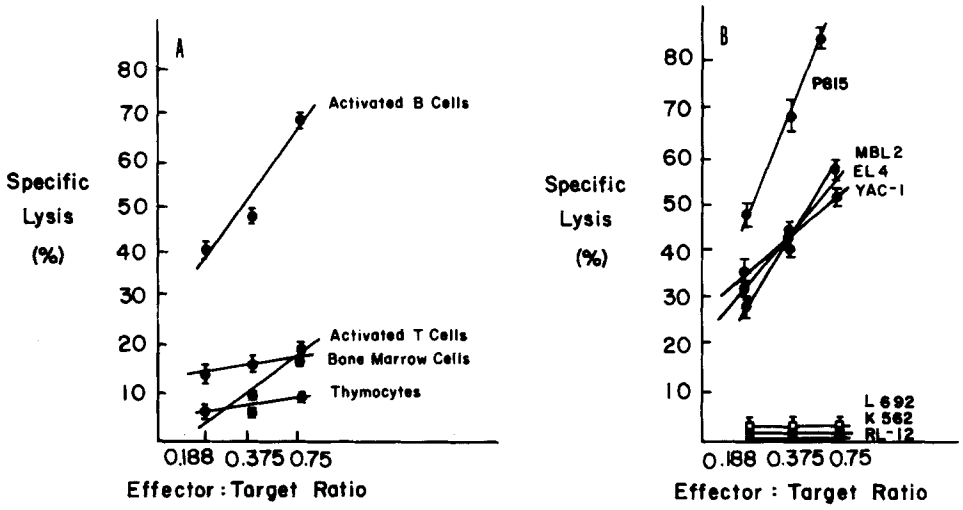

FIG. 3. Cellular targets of Cl.Ly-1$^-$2$^-$NK-1$^+$/11. Cl.Ly-1$^-$2$^-$NK-1$^+$/11 was incubated with radiolabeled cells from (A) normal tissue or (B) malignant cells for 3.5 h (see Materials and Methods).

material as determined by conventional histochemistry. Acid phosphatases were detected in lysosomal granules.

Cellular Targets of Cl.Ly-1$^-$2$^-$NK-1$^+$/11. Cl.Ly-1$^-$2$^-$NK-1$^+$/11 was tested for lytic activity against several different target cells. YAC-1 and MBL2 lymphoma cells were sensitive to lysis. In addition, P815 mastocytoma and EL4 lymphoma cells, which are

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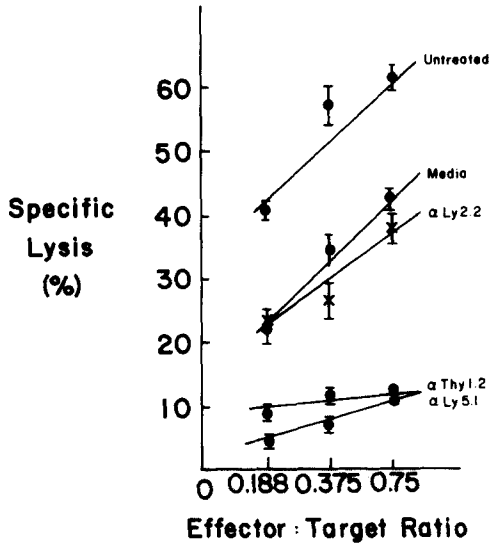

FIG. 4. Sensitivity to treatment with anti-Thy-1 and anti-Ly-5 plus complement. Cl.Ly-1$^-$2$^-$NK-1$^+$/11 cells were incubated with the indicated antisera or monoclonal antibodies, followed by complement. This treatment was repeated, and lysis of YAC-1 lymphoma was measured. Incubations with antisera alone did not reduce lytic activity.

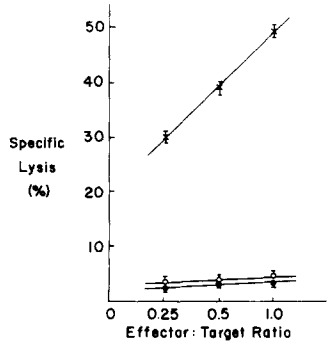

FIG. 5. ADCC to RL-12 leukemia. Cl.Ly-1$^-$2$^-$NK-1$^+$/11 was incubated with radiolabeled RL-12 mouse leukemia cells that had been incubated previously with rabbit anti-mouse lymphocyte antibody (×), rabbit anti-human lymphocyte antibody (●), or no antibody (○). Addition of either IgG fraction in the absence of cloned cells causes no lysis of RL-12 leukemia cells.

sensitive to "activated" NK cells (1, 13, 14), were also lysed. We also tested Cl.Ly-1$^-$2$^-$NK-1$^+$/11 for lytic activity against normal syngeneic target cells derived from various tissues. Syngeneic LPS-activated B lymphocytes were very sensitive to lysis in contrast to concanavalin A-activated T lymphocytes, thymocytes, and bone marrow cells (Fig. 3). NK-resistant tumors RL-12 or L692 were resistant to lysis, as was the K562 erythroleukemia, a human NK target cell. Lytic activity was eliminated after two treatments of Cl.Ly-1$^-$2$^-$NK-1$^+$/11 with anti-Ly-5 and anti-Thy-1 plus complement (Fig. 4).

Cl.Ly-1$^-$2$^-$NK-1$^+$/11 Mediates ADCC. Uncloned populations of cells that mediate certain types of ADCC copurify with NK cells (16, 17). We tested whether a cloned population bearing the glycoprotein pattern associated with NK activity could

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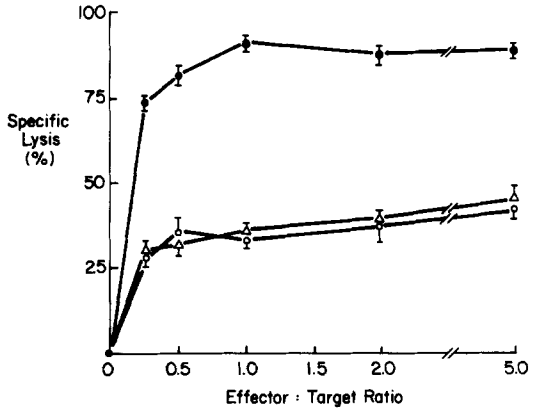

FIG. 6. ADCC with mouse antibody. Cl.Ly-1$^-$2$^-$NK-1$^+$/11 was incubated with radiolabeled LPS-activated B10.D2 B lymphocytes that had previously been incubated with the indicated antisera: △, none; ○, BALB anti-B6; ●, B6 anti-BALB.

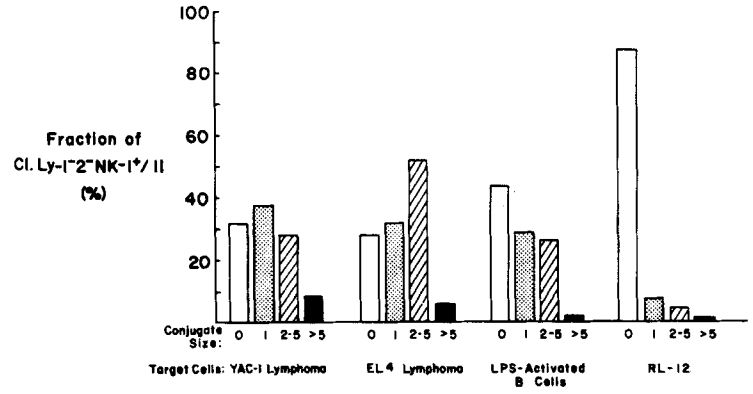

FIG. 7. Conjugate formation of Cl.Ly-1$^-$2$^-$NK-1$^+$/11 with target cells. Cl.Ly-1$^-$2$^-$NK-1$^+$/11 was incubated with rhodamine dye, washed, and mixed with the indicated target cells. Suspensions were centrifuged, and cell pellets were resuspended and transferred immediately for counting. Data are expressed as the fraction of cloned cells binding to no target cells, 1 target cell, 2-5 target cells, or >5 target cells. Cloned cells were distinguished from target cells by the presence of intracellular rhodamine label.

mediate ADCC. Cl.Ly-1$^-$2$^-$NK-1$^+$/11 cells were incubated in the presence of rabbit antibodies to mouse lymphocytes with the NK-resistant mouse leukemia, RL-12. Addition of this antibody increased lysis at effector:target ratios of 1:1 or less. No lysis was observed after incubation with immunoglobulin directed against irrelevant target cells (Fig. 5). We have also observed ADCC using mouse Ig. Addition of mouse alloantibodies directed against H-2^d targets, but not irrelevant antibodies, enhanced lytic activity against sensitive B10.D2 LPS-activated B cells (Fig. 6).

Conjugate Formation. Cl.Ly-1$^-$2$^-$NK-1$^+$/11 was internally labelled with rhodamine dye, mixed (1:10) with target cells, and the mixtures were transferred immediately for counting by fluorescence microscopy. Cloned cells formed conjugates with sensitive (YAC-1, EL4, LPS-activated B cells), but not resistant (RL-12) target cells (Fig. 7). The average conjugate size was 1 effector/2-5 target cells, although many 1:1 conjugates were observed.

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Discussion

Uncloned NK cells lyse certain tumor and normal targets and bear characteristic surface membrane antigens. We now find that cloned populations that similarly lyse various malignant and normal cells also carry the cell surface molecular labels attributed to activated NK cells. In particular, these cells display the NK-1 antigen, which has thus far been found only on NK cells. We do not know whether these cells are uniquely responsible for activated NK cell function. Data from uncloned populations suggests that several cell sets may contribute to NK activity (13–15, 30, 31). Activated NK cells appear after stimulation with a variety of agents, including partially purified IF or agents that induce IF secretion (10, 12, 13). Whether this activity reflects the differentiation of a single NK cell set or activation of an independent line of NK-related cells is unknown. Evidence from cold target inhibition studies supports the notion that both cell types recognize similar target structures (31), suggesting an ontologic relationship between these cells. In this study, Cl.Ly-1^-2^-/NK-1^+/11 has been grown in media containing IF (A. Neber, personal communication) and has properties displayed by activated NK cells.

In uncloned populations, the definition of NK activity has become increasingly obscure. Lysis of susceptible NK targets such as the YAC-1 lymphoma has been adopted as one measure of NK cell function. But most cell populations also contain cytotoxic T lymphocytes (CTL), whose activity may contribute to the lysis of lymphoma cells. These CTL, or their immediate precursors, also have been found in nude mouse strains (32). Even when antisera and complement are used to deplete CTL, single treatments may not be sufficient to lyse cells that bear a particular surface antigen (8). Therefore, we cannot completely exclude a contribution of T cells or macrophages to activated NK cell function in vivo, and we have occasionally observed Lyt-2^+ clones in vitro that lyse the YAC-1 lymphoma.

The clone described here differs from CTL because it does not bear the Lyt-2 glycoprotein by immunofluorescence and is resistant to treatment with antisera and complement. In contrast to Lyt-2^+ cell clones, the clone described here does not contain PAS-positive material. In addition, it does not show an H-2-restricted pattern of lysis, although we do not know whether H-2 molecules must be present for lysis to occur. We have also found that Cl.Ly-1^-2^-NK-1^+/11 is dependent on a factor similar to T cell growth factor for its proliferation (data not shown). By these criteria, the clone described in this report represents a line of differentiation closely related to T cells. Because Cl.Ly-1^-2^-NK-1^+/11 bears the Thy-1 and Ly-5 glycoproteins, it may belong to early cells of the thymus-dependent lineage; however, these glycoproteins are not exclusively expressed on T lymphocytes (33, 34). Unlike mature macrophages, this clone does not adhere to plastic, does not phagocytize antibody-coated erythrocytes, and does not contain the nonspecific esterase enzyme. Cells contain many vacuoles and large eosinophilic granules (Fig. 2) that do not carry peroxidase, lysozyme, or glycosylated material as determined by the PAS reaction, but they do contain the acid phosphatase enzyme. The presence of prominent lysosomal granules raises the possibility that lysosomal enzymes are involved in NK lysis. Although Cl.Ly-1^-2^-NK-1^+/11 cell lysates do not mediate YAC-1 lymphoma lysis (data not shown), additional steps involving recognition of target structures may be required for their activation.

In uncloned populations, the cells that perform NK function also express selective

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ADCC activity (17), but whether the same population can perform both functions has not been shown. We now find that Cl.Ly-1$^-$2$^-$NK-1$^+$/11 can mediate at least two specialized functions. In addition to their ability to lyse certain malignant and normal cells, Cl.Ly-1$^-$2$^-$NK-1$^+$/11 can mediate ADCC. In principle, the NK activity observed in these experiments could be mediated by ADCC because FCS contains small quantities of Ig, which might bind to target cells and mimic NK activity. We cannot presently exclude this possibility, although several observations do not support it. First, lysis did not vary according to the source of serum used in culture. Second, addition of antibody to NK-susceptible target cells increased lysis. To rigorously confirm that Cl.Ly-1$^-$2$^-$NK-1$^+$/11 NK activity is due not to ADCC, however, cells must be grown in a defined medium in the absence of serum.

We have also found that Cl.Ly-1$^-$2$^-$NK-1$^+$/11 can use mouse Ig to enhance lysis of target cells (Fig. 6). This finding, together with the observed lysis of syngeneic B cells, raises the possibility that this cell may be involved in the regulation of antibody secretion. We do not know whether lysis of LPS-activated B lymphocytes occurs through an NK mechanism or through ADCC. LPS-activated B lymphocytes may express viral determinants (35, 36), and these molecules may serve as NK target structures. Alternatively, autoantibodies or anti-idiotypes synthesized by B lymphocytes could be used to direct lysis via ADCC, which could lead to specific clonal deletion. In either case, the observation that cells of the NK type recognize and lyse activated B lymphocytes suggests that these cells may play an important role in Ig secretion.

Despite evidence that our cloned populations are homogeneous, it is possible that cells continuously differentiate into subsets in cell culture, and that this may be recognized in the future by antisera to immunogenetic markers not yet defined. We have observed similar multiple biologic activities in an inducer T cell clone (37). Because we are not certain whether the cells of Cl.Ly-1$^-$2$^-$NK-1$^+$/11 are uniform, the question whether individual cells can perform multiple biologic functions must await further investigation.

Summary

A special class of immunologic cells can lyse or damage a variety of target cells, notably malignant cells in vitro. These cells have been called natural killer (NK) cells because lysis does not require deliberate immunization by tumor cells. Although these cells can be distinguished from conventional T cells, B cells, and phagocytic cells, they have been difficult to define.

We describe a representative cloned cell line that was obtained by cloning Ig$^-$Ly-5$^+$ cells from spleen. This clone, Cl.Ly-1$^-$2$^-$NK-1$^+$/11, displays Thy-1, Ly-5, Qat-4, Qat-5, and NK-1 cell surface antigens, and lyses the NK-sensitive YAC-1 lymphoma cells, but does not lyse RL-12 cells, an NK-resistant lymphoma. In addition, this clone lysed the P815 mastocytoma, EL4 lymphoma, and lipopolysaccharide-activated B lymphocyte targets. This cloned population therefore combined information for a unique display of cell surface antigens and specialized function similar to "activated" NK cells. Because this cloned population forms conjugates with susceptible but not resistant target cells, it may prove useful to identify the structure of cell surface molecules that recognize foreign cells. Finally, cells of this clone also specifically lysed target cells coated by antibodies to determinants on the target cell surface, demon-

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strating that a single cloned cell population can mediate two specialized immunologic functions: antibody-dependent cellular cytotoxicity and NK cell lysis.

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