KILLER CELLS: A FUNCTIONAL COMPARISON
BETWEEN NATURAL, IMMUNE T-CELL
AND ANTIBODY-DEPENDENT IN VITRO SYSTEMS*

BY ROLF KIESSLING, GYÖZÖ PETRANYI,† KLAS KÄRRE, MIKAEL JONDAL, DANIEL
TRACEY,§ AND HANS WIGZELL

(From the Department of Tumor Biology, Karolinska Institute, 104 01 Stockholm 60, Sweden and
the Department of Immunology, Uppsala University, Uppsala, Sweden)

Evidence is accumulating for naturally occurring humoral and cellular immunity against mouse C-type particles or mouse tumor cells. (1–9 and footnote 1). It has recently been shown in several systems that cell-mediated immune reactions, as measured by in vitro killer cell activity, occur naturally among rodents (2, 4, 8, 9, 10, and footnote 1). We have described the existence of natural killer (NK) cells against certain mouse lymphomas, notably of Moloney type (10). This NK activity could be shown to be under polygenic control, with a major role being played by H-2 locus-associated gene(s) (11). A high degree of concordance was seen when syngeneic or semisyngeneic mice of different genotypes were compared for in vitro NK activity and for in vivo resistance to Moloney lymphoma cells (12). In fact, there are reasons to believe that these NK cells may play one of the most important roles in the initial resistance against transplantation of syngeneic Moloney lymphomas in the mouse (13).

This NK activity seemed to be specific in the sense that only certain lymphoma target cells were killed, whereas others, although highly sensitive for immune T-cell killing, were unaffected. When a limited number of cell lines were studied the specificity seemed to be restricted to Moloney leukemia virus-induced lymphomas (10), but recent results show that also certain other types of tumors might be sensitive (unpublished results).

Spleen cells from congenitally athymic nude mice have high NK activity (14) which provided the first indication of a non-T-cell nature of the killer cell.

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† Fellow of UICC. Present address: National Institute of Haematology and Blood Transfusion,
Budapest, Hungary.
§ Fellow of the Jane Coffin Childs Memorial Fund for Medical Research. Present address: The
Division of Immunology, The Good Samaritan Hospital, Baltimore, Md.
1 Nunn, M. E., J. Y. Djeu, M. Glaser, D. Lavrin, and R. B. Herberman. 1975. Natural cytotoxic
reactivity of rat lymphocytes against syngeneic Gross virus-induced leukemia. Submitted for
publication.
2 Abbreviations used in this paper: ADCC, antibody-dependent cellular cytotoxicity; CRBC,
chicken red blood cell; CTL, cytotoxic T lymphocytes; EAC, erythrocyte antibody complement;
NK, natural killer (cells).
Further strengthening its non-T-cell nature, the NK cell proved to be resistant to antitheta plus complement treatment. However, it is not a B cell inasmuch as depletion of Ig-positive cells only served to enrich for killer cell activity (14). Thus, this cell lacks the surface characteristics of mature T or B lymphocytes. Still its low adherent properties as well as its morphology (14), make it highly probable that the NK cell is a lymphocyte. Furthermore, preliminary data suggest the NK cell to be distinct from the K cells participating in antibody-induced cell-mediated (ADCC) reactions (14). In all, the characteristics of the NK cell seem very similar to those of the N cell described by Herberman et al. in another NK cell system (15).

In the present article we have tried to further define the characteristics of the NK cells in comparison to two other previously defined killer cell systems.

**Materials and Methods**

*Mice.* 1-2-mo-old CBA mice maintained by continuous single-line brother-sister mating in our laboratory were used.

*Tumors.* The YAC line was derived from a Moloney virus-induced lymphoma in A/Sn mice and propagated in ascites form. An in vitro subline, YAC-1, adapted to stationary suspension culture (16) was maintained in medium F 13.

Also the methylcholanthrene-induced P 815 mastocytoma of DBA/2 origin was used either as an in vitro line or propagated in the ascites form.

**Preparation of Effector Cells.** Spleen cell suspensions were prepared in F 13 medium completed with 10% fetal calf serum and HEPES buffer (pH 7.3).

Peripheral blood leukocytes were prepared from heparinized whole blood using a Ficoll-Isopaque gradient (17). In some experiments adherent and phagocytic cells were removed by carbonyl iron and magnet treatment (17).

**Immunization Procedure.** CBA mice were immunized once with 3 x 10^7 living P 815 ascites cells i.p. 9-12 days after immunization their spleen cells were used as source of immune anti-P 815 cells.

**Cytotoxic Assays in the NK, P 815, and Chicken Red Blood Cell (CRBC) Systems.** A conventional 6 h ^51^Cr release technique was used in the NK, P 815, and CRBC systems, as previously described (14), using triplicates. Experimental details like pretreatment of effector cells, incubation procedures, and incubation time was always identical between the three systems, and experiments were always run in parallel.

In the CRBC system a mouse antichicken erythrocyte or a normal mouse serum at a 10^4-fold dilution was added. Isotope release of the CRBC cells in the presence of medium alone was always identical to isotope release in the presence of normal mouse serum, and therefore these data are not shown. In the P 815 system normal spleen cells were not included as controls, since in our hands this tumor has proved to be resistant to lysis by normal spleen cells (10). Isotope release is expressed as percent lysis according to the previously described formula (10).

**Erythrocyte Antibody Complement (EAC) Rosette Technique.** EAC rosettes were prepared and separated as previously described (17).

**Trypsin Treatment of Effector Cells.** Spleen cells at a concentration of 2-3 x 10^7 ml were treated with different concentrations (see figure legends) of trypsin (State Bacteriological Co., Stockholm, Sweden) for 45 min at 37°C and thereafter washed twice in complete F 13 medium.

**Preparation of Aggregated Gamma Globulin.** Human or mouse IgG from normal serum was produced by standard DEAE-cellulose and Sephadex G-200 fractionation procedures. IgG of a concentration of 10 mg/ml in phosphate-buffered saline, pH 7.4, was heat aggregated using 10-min incubation at 63°C.

**Nylon Wool Column Passage.** Normal or immune CBA spleen cells were passed through a nylon wool column according to a modification (18) of the original method (19).

**Preparation and Use of Antitheta Serum.** ARR anti-C3H antiserum was produced and used as previously described (14).
Results

Separation of complement Receptor-Bearing Cells with EAC Rosette Technique. Most B cells, as well as cells of the monocytic series, carry C3b or C3d complement receptors (17). The EAC rosette fractionation technique is a sensitive method for selective fractionation of these receptor-bearing cells.

In our hands this technique gave a more complete separation and caused less nonspecific cell loss when peripheral blood was used instead of spleen as lymphocyte source. Peripheral blood leukocytes are more active in the NK system than spleen cells and show the same genotype dependency. Like spleen cells, they are resistant to iron-magnet treatment and to treatment with antitheta serum and complement (results not shown here). We thus conclude that the same NK cell was active in both of these cellular compartments.

In four separate experiments using EAC rosette depletion techniques we could show that the depleted cells were equally or somewhat more active than the nonseparated cells in lysing the YAC cells (see example in Fig. 1 a). Thus a cell population without complement receptor-positive cells is highly efficient in the NK system. In contrast, but as expected (20), EAC depletion strongly reduced the killer efficiency in the ADCC system (Fig. 1 b), which further supports the notion of the nonidentity of these two systems.

Fractionation of Spleen Cells with the Nylon Wool Column Method and with Antitheta Serum Plus Complement. The low adhesiveness of the NK cell has previously been shown with the iron-magnet method. Also, the non-B-cell character of this cell type was suggested by the failure to be removed via passage of spleen cells through an antimouse Ig column, which if anything caused an increase of NK activity of the passed cells (14). In line with the results from both these methods the nylon wool column passage method, known to remove adherent cells and B cells (19), failed to diminish the NK activity of the passed spleen cells in any of four experiments performed. If cells sticking to nylon fibers were mechanically eluted by stirring with a Pasteur pipette, these cells were clearly less efficient as killer cells against YAC-1 than nonpassed or passed cells (Fig. 2 a).

Similar results were obtained in the P 815 immune system, where passed spleen cells were as cytotoxic as nonpassed against P 815 target cells (Fig. 2 b).

![Figure 1](image-url)  
**Fig. 1.** Effect of EAC rosette depletion on the cytotoxicity in the NK (a) or CRBC system (b). EAC rosette-depleted (○ – ○) or nondepleted (x – x) peripheral blood leukocytes from normal CBA mice were used as effector cells.
Contrariwise to the results from these two systems the killer efficiency in the CRBC system was always clearly, if not totally, decreased by passage (Fig. 2 c).

The NK cell has been shown to be insensitive to treatment with antitheta serum and complement. Furthermore, if antitheta serum treatment was followed by passage through an anti-Ig column, this combined fractionation method only served to enrich for killer cell activity (14). Similar experiments were now performed with a nylon column instead of an anti-Ig column. Although the enrichment of NK activity was not as pronounced with this fractionation procedure, the remaining cells were indeed highly cytotoxic for the YAC-1 target cells (Fig. 3 a). In contrast, after treatment with antitheta serum and complement, the immune anti-P 815 activity was totally abrogated as expected (Fig. 3 b).

These results thus confirm that the dominant cell population active in the NK system is low adherent and lacks theta antigen as well as surface immunoglobulin. They do not rule out, however, that minor nonimmune cytolytic effects could be attributed to T cells, B cells, or monocytes.

Killer Cell Activity after Addition of Aggregated Human or Mouse Gamma Globulin. Fig. 4 illustrates the effect of adding heat-aggregated human or mouse immunoglobulin to the NK, CRBC, or P 815 systems. The mouse immunoglobulin was either prepared from normal serum of CBA mice (high reactive in the NK system) or ACA mice (low reactive in the NK system). No difference in the activity of the immunoglobulin according to strain of origin was observed.

As expected, a pronounced inhibition in the CRBC system was seen with both the human and mouse preparations at dilutions down to 1/3125 (Fig. 4 c). No significant inhibition was seen in the NK system (Fig. 4 a) or in the P 815 system (Fig. 4 b) even at the higher concentrations. This result thus strongly argues against the NK system to rely on killer cell mechanisms involving activation of lymphocytes through Fc receptors.
FIG. 3. Effect of combined anti-θ + complement treatment followed by passage through nylon wool column on the cytotoxic effect in the NK system (a). As control the effect of anti-θ complement treatment in the immune P 815 system is shown (b). Normal (a) or immune (b) CBA spleen cells treated with normal mouse serum and complement (××), anti-θ serum plus complement (△△), or anti-θ serum plus complement followed by nylon wool column passage (□□) were used as effector cells.

FIG. 4. Effect of heat-aggregated gamma globulin on the cytotoxicity in the NK (a), P 815 (b), or the CRBC system (c). Percent lysis by normal (a and c) or immune (b) CBA spleen cells alone at an effector/target cell ratio of 50/1 is indicated (---). Human (××) or mouse gamma globulin from ACA (○○) or CBA (△△) mice were used. In the undiluted preparation (1/1) the concentration of aggregated gamma globulin was 160 mg/ml for the human and 20 mg/ml for the mouse in the final incubation mixture.

Trypsin Treatment of Effector Cells. Proteolytic enzymes digest most cell surface receptors, except for Fc receptors (21). Therefore pretreatment of effector cells with trypsin might distinguish Fc receptor dependent from Fc receptor independent immune reactions. In a series of experiments the resistance of spleen cells to pretreatment with trypsin at various concentrations was investigated. After trypsin treatment spleen cells were tested simultaneously in the NK, CRBC, and the immune P 815 systems. The results from one such representative parallel experiment is illustrated in Fig. 5. As shown earlier by others (22), the effector cell in the ADCC system was relatively trypsin resistant, although a slight decrease in killing activity started to appear at a concentration
of 0.25% trypsin, being still more marked at 1% (Fig. 5 c). When the same spleen cells were tested for NK activity against YAC-1 cells a strong reduction already at 0.25% trypsin concentration was noted (Fig. 5 a). This reduction was comparable to the effect after similar treatment of immune spleen cells in the P 815 system (Fig. 5 b). Low trypsin concentrations (0.001%) did not markedly affect any of the three systems (Fig. 5). Thus, both NK cells and CTL were quite sensitive to trypsin treatment in comparison to the effector cell system involved in ADCC reactions.

Discussion

In cell-mediated immune responses to allografts and tumors cytolytic thymus-derived lymphocytes are known to play an important role (for references see 23). In contrast to this, we and others have previously shown that nonimmune, so called, NK cells are insensitive (14) or almost insensitive (15) to antitheta serum treatment. These data and the fact that nude spleen cells are highly efficient as killer cells would argue against this cell type being a mature T cell. The possibility that the NK might be a precursor T cell not expressing Thy-1 antigen has, however, been considered by us. Pretreatment of normal or nude mice in vivo or in vitro with various nonthymic (polyadenylic-uridylic acid, lipopolysaccharide) or thymic agents, by others reported to induce theta positiveness in T-precursor cells (24), has so far not rendered the NK cell sensitive to antitheta treatment. We are now continuing this search for a surface marker on the NK cell, also using various antisera directed against differentiation antigens on the precursor T cells.

NK cells have previously been found to lack detectable surface Ig (14, 15), and in the present article they could be shown also to lack receptors for C3 components. Similar results were seen by others using EAC monolayer absorption
Contrariwise, similar treatment would cause an almost total abrogation of killer activity in the ADCC system.

In the present article we also tried to further analyze the means through which NK cells do exert their cytolytic activity. We could confirm the results of others (most likely studying the very same cell type [15]) that aggregated IgG, while completely abrogating the cytotoxicity in ADCC displayed no detectable impact on the NK activity. These data strongly argue against any cytophilic antibody being present on the NK cells and thereby inducing these cells to become killer cells.

We have previously found the NK cells to be nonadherent as measured by carbonyl iron and magnet and/or passage through glass bead columns (14). Other workers have reported similar findings (15). In the present article we now report on further work using nylon fiber columns known to be highly efficient in the removal of adherent cells (19). Both adherent and nonadherent cells were tested for their capacity to function as NK cells. Again, the nonadherent cells contained excellent activity, whereas the adherent, eluted cells displayed considerably weaker NK function. We would thus conclude that under three varying conditions, each of which are known to remove adherent cells, NK cells failed to adhere to any significant degree.

The trypsin sensitivity of the NK activity was also analyzed in parallel to the ADCC and the CTL systems. Here, it was found that both NK and T-killer cells were quite trypsin sensitive, whereas the ADCC effector cells were relatively trypsin resistant. This would fit nicely with earlier reports on the Fc receptor involved in ADCC to be trypsin resistant (22) and would then further dissociate the NK cell from the K cell in the ADCC systems. However, Herberman et al. found the NK cell to be trypsin resistant (15). This discrepancy with our results could probably be explained by their use of a considerably shorter trypsin preincubation period (20 min compared to 45 min).

We are then left with somewhat of a dilemma. The NK cells look like small lymphocytes (the population left from mouse spleen cells after removal of adherent cells and T and B lymphocytes is to more than 95% small lymphocytes and contain virtually all NK activity [14]). Yet, no surface marker characteristic of either T or B lymphocytes or of monocytes has been found on the NK cell surface. Furthermore, the NK cells express cytotoxic activity with specific direction analogous to conventional immune reactions indicating an "inborn" reactivity carried out by products of the NK cell itself. However, it still remains to determine the nature of those structures on NK cells which endow these cells with their immune (or immune-"like") specificity. Such a search would however seem very worthwhile as the present NK activity seems to represent a major (maybe the major) barrier in the resistance towards certain syngeneic or semi-syngeneic virus-induced lymphomas in the mouse (13).

Summary

Previous reports have shown that spleen cells from nonimmune adult mice of certain strains do regularly kill Moloney leukemia virus-induced lymphomas in short-term $^{51}$Cr release assays. This naturally occurring killer (NK) cell had low adherent properties and had the morphological appearance of a lymphocyte. Still it lacked surface characteristics of mature T or B lymphocytes.
In the present report a functional study was carried out, comparing in parallel the NK system, the T-cell killing across an H-2 barrier (anti-P815), and the antibody-dependent cell-mediated chicken red blood cell (CRBC) system. In contrast to the effector cells in the CRBC system, the NK cells were insensitive to erythrocyte antibody complement (EAC) rosette depletion and would pass through nylon wool columns. NK activity was not inhibited by the presence of heat-aggregated human or mouse gamma globulin, in contrast to the strong inhibition noted in the CRBC system. Sensitivity to trypsin pretreatment was noted in the NK system as well as in the immune P815 system, whereas the CRBC system was relatively trypsin resistant. Antitheta plus complement eliminated the anti-P815 activity, but did not touch the NK activity. The present results thus further distinguish the NK cell from cytotoxic T lymphocytes or from antibody-dependent killer cells.

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