Proc. Natl. Acad. Sci. USA
Vol. 78, No. 10, pp. 6396–6400, October 1981
Immunology

Selective natural killer resistance in a clone of YAC lymphoma cells

(tumor variants/natural killer cells)

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Communicated by George Klein, June 1, 1981

ABSTRACT **YAC lymphoma cells were treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and then cloned and subcloned. Of 51 clones, 3 were selected for further study. Tenfold more natural killer (NK) effector cells were required to lyse YAC clone 6 and subclone 6-28 cells compared with clone 19 cells or the YAC parent cell line. The maximum plateau level of cytolysis of the NK-resistant (NK^R) variants (20%) never approached that of the NK-sensitive (NK^S) variants or YAC parental cells (60%) even after prolonged incubation (20 hr). NK^R variants appeared with equal frequency (0.10) on cloning YAC cells that had not been treated with mutagen but these variants were highly unstable with respect to NK sensitivity and were not studied further. Cytolysis of both NK^R and NK^S lines was mediated by nylon-nonadherent asialo-GM1$^+$ effector cells, and effectors from poly(I)·poly(C)-boosted mice preferentially lysed the NK^S lines. The NK^R alteration did not appear to change the NK target structure (NK-TS): (*i*) unlabeled NK^R cells competed equally with NK^S cells in reciprocal unlabeled-target competition assays; (*ii*) the frequency of target–effector conjugates was identical with NK^R or NK^S lines; and (*iii*) normal rabbit serum, which contains antibodies thought to react with the NK-TS, reacted equally against both NK^R and NK^S targets. The NK^R alteration was selective for NK cells and did not result in a resistance to lysis in general; NK^R and NK^S variants were equally susceptible to (*i*) cytolysis mediated by alloimmune or lectin-dependent effector T cells and (*ii*) antibody- and complement-mediated lysis. These results are compatible with the hypothesis that the NK^R variants have an altered acceptor site on the target cell membrane that normally binds the "lytic moiety" delivered by the effector cell.**

One of the key questions in the study of natural killer (NK) cells concerns the properties of the target cell that render it susceptible to cytolysis (1). Although the target structure that is recognized by NK cells (NK-TS) has been isolated (2) and the expression of NK-TS is known to be a necessary condition for cytolysis (3, 4), there are undoubtedly other factors involved in this complex series of events, such as susceptibility to interferon-mediated protection (5) and membrane hydrophobicity (6). Another possible target cell feature that could influence its sensitivity to cytolysis would be the presence of a putative acceptor for the NK "lethal hit" moiety, as schematized in ref. 3. To test this possibility, we set out to derive variants of YAC cells that might exhibit independent alterations in either the NK-TS or the putative acceptor site.

The method chosen for mutagenesis was based on studies by Boon and Kellermann that showed that a high frequency (10%) of stable antigenic variants could be obtained from mouse tumors by a combination of mutagenesis and cloning (7). We report here that some clones derived from YAC after mutagen treatment were selectively more resistant to NK-mediated lysis but did not exhibit a marked alteration in NK-TS expression. This finding is used to develop a model that postulates the existence of an acceptor on the target cell for a lytic moiety delivered by the NK cell.

MATERIALS AND METHODS

Animals. Six- to ten-week-old mice were used in all experiments and all groups were strictly age matched. CBA/J, C57BL/6J, and A/J mice were obtained from The Jackson Laboratory.

Tumor Cell Lines. Tumor cell lines were maintained by continuous *in vitro* culture. YAC is a T-cell lymphoma induced by Moloney leukemia virus in A/sn mice, RBL-5 is a RadLV-induced T lymphoma from C57B1/6 mice (both lines generously provided by Rolf Kiessling, Stockholm), and P815-2 is a mastocytoma originally induced in DBA/2 mice by methylcholanthrene.

Mutagenesis and Cloning. YAC cultures were treated for 1 hr at 37°C with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) at 5 μg/ml in dimethyl sulfoxide, washed, and cultured for 6 days to allow any mutation to segregate. This dose of mutagen killed 90% of the YAC cells as determined by trypan blue exclusion. Cultures were then cloned by limiting dilution in Terasaki plates (10 μl per well). Wells containing only one cell (1/3 of all wells) were confirmed by visual inspection. When colonies of ≈30 cells grew up, they were transferred to Petri dishes and maintained in bulk cultures. One of the original clones, YAC-6, was subcloned by using the same procedures.

Nylon Columns. Monodispersed spleen cells were treated for 4 sec with H_2O to remove erythrocytes by hypotonic shock, and the remaining cells were passed over nylon wool columns; cell recoveries were 10–20% of input.

Anti-Asialo GM1. Spleen cells (20×10^6/ml) were treated for 30 min at room temperature with rabbit anti-asialo GM1 serum (1:40) (generously provided by Ko Okumura, University of Tokyo, Japan) and then for 45 min at 37°C with guinea pig complement (1:5), as described by Kasai *et al.* (8). Viable cell recovery was 80–90%.

Alloantisera. Monoclonal mouse anti-H-2K^k antibody (Becton Dickinson, Mountain View, CA), monoclonal mouse anti-thy-1.2 antibody (New England Nuclear), and conventional mouse anti-Ly 5.1 antisera (kindly provided by Ian F. C. McKenzie, Melbourne) were tested against YAC cells by using a standard two-stage complement-dependent cytotoxicity test. Normal rabbit serum (1:12) pre-absorbed with YAC cells was used as a source of complement. Dead cells were determined by trypan blue uptake using normal mouse serum as control:

$$\%\ \text{dead cells} = \frac{\%\ \text{cells lysed (antiserum)} - \%\ \text{control dead cells}}{100 - \%\ \text{control dead cells}}.$$

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Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; NK, natural killer (cell); NK-TS, NK target structure; NK^R and NK^S, NK resistant and NK sensitive, respectively; Con A, concanavalin A.

Chromosomal and DNA Analysis. Chromosome counts were performed on Giemsa-stained chromosome spreads. Colcemid (GIBCO) was used as the mitotic blocking agent. DNA content was assessed by flow microfluorometry using propidium diiodide as stain. Approximately 2×10^4 cells were analyzed by a Coulter EPICS multiparameter sensor, viable sample-handling system, and model SPA single-parameter analyzer.

Mixed Lymphocyte Culture. Responder spleen cells were cultured in 50-ml stationary Falcon flasks at 10^6/ml in 10 ml of alpha medium/15% fetal calf serum/5 mM Hepes/20 μM 2-mercaptoethanol. Stimulator spleen cells (10^7/ml) were treated for 1 hr at 37°C with mitomycin C at 100 μg/ml and then extensively washed. Similar conditions were used for the generation of lectin-dependent effector cells.

Target-Binding Cell Assay. Nylon wool-passed spleen cells were labeled for 10 min at room temperature with fluorescein diacetate at 5 μg/ml per 10^6 cells. Only viable cells are labeled by this procedure. Labeled lymphoid cells (2×10^5) were mixed with 10^6 target cells in 0.5 ml of culture medium and centrifuged at $200 \times g$ for 5 min at room temperature. The tubes were placed on ice for 30 min and then aspirated five times with a Pasteur pipette. The percent fluorescing cells (effectors) binding to nonfluorescing cells (targets) was then determined under a UV microscope.

Cytolytic Assay. Target cells (1×10^4), labeled with ^{51}Cr (sodium chromate) as described (9), were incubated for 3 hr at 37°C with various numbers of effector cells in 0.2-ml microwells (Linbro) in triplicate or quadruplicate samples. The microplates were then centrifuged and 75 μl of supernatant was measured in a γ-ray counter. Spontaneous release was determined by culturing ^{51}Cr-labeled targets alone, and total label was determined by assaying an aliquot of target cells after resuspension in the microwells. The following formula was used to compute % lysis:

$$\% \text{ lysis} = \frac{\text{test cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100.$$

Reagents. Stock solutions consisted of fluorescein diacetate (Sigma) in acetone diluted in medium to 5 μg/ml. Poly(I)·poly(C) (Miles) was dissolved in H_2O to 1.0 mg/ml. MNNG (Aldrich) was dissolved in 10% dimethyl sulfoxide and diluted in medium prior to use.

RESULTS

Selection of NK^R Mutants. YAC cells maintained by continuous *in vitro* culture were treated with MNNG and cloned by limiting dilution. Fifty-one clones were grown up and tested for NK sensitivity in a ^{51}Cr release assay; as shown in Fig. 1, reproducibly, clone 6 was the lowest and clone 19 was the highest in NK sensitivity to nylon wool-passed spleen cells. Clone 6 was recloned and, of the 28 subclones subsequently grown up, clone 6-28 was the lowest in NK sensitivity. It was found that NK lysis of the YAC parent and clone 19, as well as residual lysis of clones 6 and 6-28, was eliminated by previous treatment of the nylon-passed spleen cells with rabbit anti-asialo GM1 and complement (Fig. 1), which suggests that the same asialo GM1$^+$ effector cell type is mediating lysis of the different clones. If the data shown are converted to lytic units/10^7 cells calculated at the 25% level of lysis, then the YAC parent and clone 19 yielded values of 85 and 100, respectively, whereas clone 6 and subclone 6-28 yielded values of 10 and 5, respectively. This represents a 10-fold difference between the resistant and susceptible lines with respect to NK sensitivity on a per-cell basis. As shown in Table 1, for NK cells that were highly boosted by poly(I)·poly(C), these differences in susceptibility were also evident. However, in terms of lytic units, the relative difference between susceptible and partially resistant lines was only 4-fold against poly(I)·poly(C)-boosted effectors compared with a 10-fold difference against nonboosted effectors. In further experiments (not shown), lysis of clone 6 and subclone 6-28 by unfractionated spleen cells was also significantly less than lysis of clone 19 or the YAC parent even after 20 hr of incubation and, therefore, it is unlikely that NK resistance by the YAC variants merely reflects a kinetic difference in cytolysis. These clones maintained this pattern of NK sensitivity for >1 year in culture. Analysis of another 50 clones from YAC that had not been treated with mutagen showed an equal frequency (0.10) of partially (5-fold) NK^R clones but these clones were highly unstable and returned to parental levels of NK sensitivity within 6 weeks (unpublished data). Therefore, only the mutagen-derived clones were studied further.

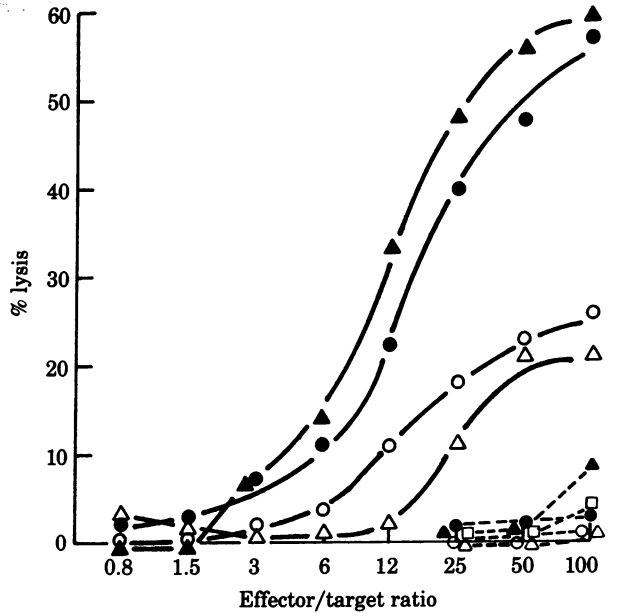

FIG. 1. Relative resistance of clone 6 and subclone 6-28 to cytolysis by enriched asialo GM1$^+$ NK populations. Spleen cells pooled from six CBA mice were passed over nylon wool columns and tested in a 6-hr ^{51}Cr release assay for cytolysis of YAC cells. This experiment was repeated six times with similar results. —, Cells previously treated with guinea pig complement (1:5); ---, cells previously treated first with rabbit antiasialo GM1 (1:50) and then with guinea pig complement (1:5). This experiment was repeated twice with similar results. Values represent mean % lysis in triplicate wells. ●, YAC parent; ▲, YAC-19; ○, YAC-6; △, YAC-6-28; □, P815.

Nature of NK Resistance. Two kinds of experiments were performed to determine whether partially NK^R YAC clones sim-

Table 1. Susceptibility of YAC variants to lysis by poly(I)·poly(C)-boosted NK cells

Target	Poly(I)·poly(C) Without	Poly(I)·poly(C) With
YAC parent	100 ± 12	1000 ± 97
Clone 19	110 ± 5	2050 ± 163
Clone 6	12 ± 2	150 ± 13
Subclone 6-28	11 ± 2	230 ± 41

Mice were untreated or injected intravenously 24 hr before assay with 100 μg of poly(I)·poly(C). Spleen cells pooled from three mice in each group were passed over nylon wool columns and tested in a 6-hr assay for cytolysis of ^{51}Cr-labeled YAC targets. Values represent mean lytic units per 10^7 cells ± SD at 25% lysis calculated from six-point effector titration curves. This experiment was repeated twice with similar results.

ply expressed less of the relevant target structure.

In target competition experiments, it was found that unlabeled target cells from the partially NK^R clones 6 and 6-28 were similar to unlabeled YAC parent cells in inhibiting NK-mediated lysis of ^{51}Cr-labeled NK^S clone 19 or YAC parent on a per cell basis (Fig. 2 *A* and *B*). Clone 19 was the most efficient competitor, whereas P815-2, an NK-insensitive target, did not compete at all at competitor/target ratios <5:1. The partially NK^R clones 6 and 6-28 were more sensitive to competition in general, which may be related to the lower level of cytolysis in control wells lacking competitors. The competitive effectiveness of NK^S or NK^R targets, however, was similar.

These results were confirmed by directly counting the frequency of lymphocytes forming conjugates with the various target cells. As shown in Table 2, nylon-passed spleen cells were equally capable of binding the YAC parent or clones 19, 6, and 6-28, whereas the NK-insensitive target P815-2 was bound to a lesser extent (4% vs. 22%). Most (75%), but not all, target-binding cells have previously been shown to represent cytolytically active NK cells (3, 4) but the method is subject to a 10–20% variation and would not be expected to detect subtle changes in the target structures recognized by NK cells.

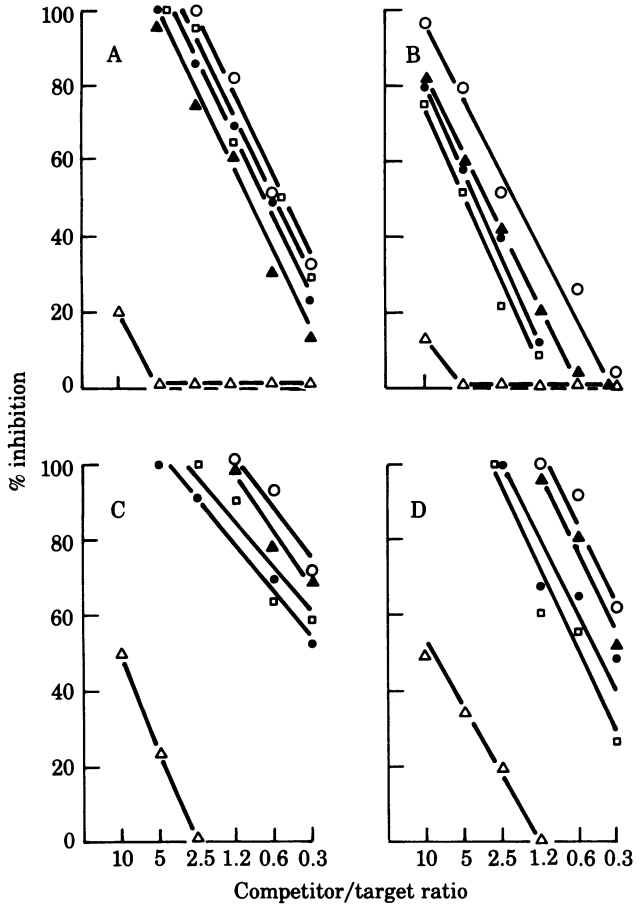

FIG. 2. Crosscompetitive inhibition by NK^R and NK^S variants. Spleen cells pooled from 10 CBA mice were passed over nylon wool and tested in a 6-hr ^{51}Cr release assay at an effector/target ratio of 40:1 against labeled targets: (*A*)YAC parent. (*B*)YAC-19. (*C*)YAC-6. (*D*)YAC-6-28. Control lysis values were 47% for YAC parent; 55% for YAC-19; 22% for YAC-6; and 18% for YAC-6-28. Unlabeled competitor cells were added to some wells at the indicated competitor/target ratio; values represent the means for triplicate wells. Competitors: ●, YAC parent; ○, YAC-19; ▲, YAC-6; □, YAC-6-28; △, P8152. This experiment was repeated three times with similar results.

Table 2. Target–effector conjugate formation with YAC variants

Target	Target-binding cells, %	Cells counted, no.
YAC parent	20 ± 3	522
YAC-6	23 ± 2	543
YAC-6-28	24 ± 3	510
YAC-19	22 ± 1	600
P815-2	4 ± 2	750

Nylon-passed CBA spleen cells were labeled with fluorescein diacetate, mixed with a 5-fold excess of target cells, centrifuged, and resuspended after a 30-min incubation on ice. Results represent mean ± SD of triplicate determinations.

Selectivity of NK Resistance. The experiments described above showed that the relative resistance of YAC clones 6 and 6-28 to NK-mediated cytolysis was not likely due to lack of expression of the NK-TS. It was then relevant to ask whether apparent NK resistance simply reflected a general resistance to any form of lysis.

Alloimmune effector cells from 5-day mixed lymphocyte cultures were equally efficient at lysing YAC parent cells and clones 19, 6, and 6-28. This was apparent when C57Bl/6 spleen cells educated against either $H\text{-}2K^dD^d$ (not shown) or $H\text{-}2K^kD^d$ (Fig. 3*A*) stimulator cells treated with mitomycin C were used. Nonstimulated effector cells were ineffective (<10%) and lysis was H-2 specific; targets showing the appropriate H-2 (P815-2, $H\text{-}2K^dD^d$) were lysed whereas H-2-irrelevant targets (RBL-5, $H\text{-}2K^bD^b$) were not. Effector cells were also thy 1.2^+ (data not shown). In further studies, effector cells were generated in 4-day spleen cell cultures containing concanavalin A (Con A) at 10 μg/ml. These effectors were equally efficient at lysing YAC parent and clones 19, 6, and 6-28 target cells in the presence of Con A (Fig. 3*B*). Control cells cultured and assayed in the absence of Con A did not exhibit significant cytotoxicity (<5%). Similar results were obtained when *in vivo*-generated lectin-dependent killer T cells were used [in this case, anti-asialo GM1/

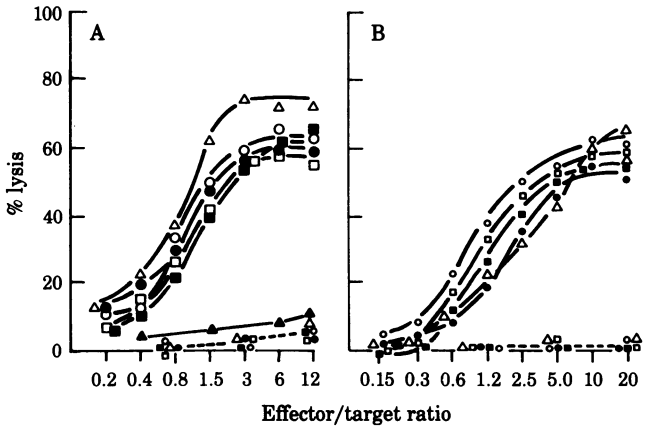

FIG. 3. Sensitivity of YAC mutants to alloimmune and lectin-dependent effector cells. (*A*) Spleen cells pooled from five C57BL/6 mice were cultured 5 days at a responder/stimulator ratio of 5:1 in the presence of mitomycin C-treated spleen cells from A/J mice (—). Control cultures were incubated in the absence of stimulators (---). Cultures were harvested, centrifuged on Ficoll to remove dead cells, and tested in a 6-hr ^{51}Cr release assay for cytolysis of YAC parent cells ($H\text{-}2K^kD^d$) (○), clone 19 (●), clone 6 (□), clone 6-28 (■), P815-2 ($H\text{-}2K^dD^d$) (△), or RBL-5 ($H\text{-}2K^bD^b$) (▲). (*B*) Spleen cells from C57BL/6 mice were cultured 4 days in the presence of Con A at 10 μg/ml (—) or in its absence (---). Cultures were harvested and tested in a 6-hr ^{51}Cr release assay in the presence of Con A at 2.5 μg/ml. Values represent means for triplicate wells. Cytolysis was reduced 85% by previous treatment of effectors with antithymocyte serum and complement. This experiment was repeated three times with similar results.

guinea pig complement treatment was used to remove interfering NK cytolysis (unpublished observation)].

In additional experiments on the selectivity of the NK^R alteration, it was found that YAC parent and clones 19, 6, and 6-28 cells were equally susceptible to lysis by antibody and complement (Table 3).

Other Features of the YAC Variant Clones. Recent studies have shown the presence of a natural antibody in normal rabbit serum that exhibits a specificity pattern similar to that of NK cells (10) and may react with the NK-TS. In the present study, NK^R clones 6 and 6-28 were equally sensitive to normal rabbit serum compared with the YAC parent or NK^S clone 19 (Table 3). This observation is compatible with the suggestion that NK resistance in the variant clones is not necessarily linked to the expression of the NK-TS.

In other experiments (Table 3), neither the YAC parent or three mutant clones expressed FcR or C3R as detected by a sensitive rosetting technique (11). In addition, there was no major difference in susceptibility of the YAC mutants to growth inhibition by wheat germ agglutinin (Table 3). The parental tumor and all three mutant clones were relatively resistant to concentrations of wheat germ agglutinin that were extremely toxic to other tumor cell lines, such as MDAY, tested in parallel (12). Normal growth characteristics, however, were correlated with NK sensitivity. Hence, the NK^R clones YAC-6 and YAC-6-28 grew at a slower rate and to a lower saturation density than the NK^S clone YAC 19 or the YAC parent (Table 3). Chromosome numbers were not significantly different in the four cell lines. In addition, there were no minute or metacentric chromosome markers observed in any of the preparations, overall chromosome morphology was normal, and the DNA contents of the various cell lines were similar as measured by the DNA intercalating dye propidium iodide. Only values for cells in G_1 phase of the cell cycle are reported to minimize the effects of different growth characteristics. Other workers, using fresh, rather than long-established tumor cell lines, have found much greater variability in G_1 phase DNA content in various clones (13).

DISCUSSION

We have studied YAC lymphoma variants that are partially but selectively resistant to cytolysis mediated by NK cells. These NK^R variants were cloned and subcloned and were found to be stable over the 1-year period of this study. The NK^R and NK^S clones and the YAC parent all exhibited a near normal diploid karyotype with a mode of 41 chromosomes and similar DNA contents. Mutagen treatment did not increase the frequency of NK^R clones but was necessary to obtain stable lines with respect to the NK^R phenotype. We speculate that the mutagen may act on some secondary target (e.g., a processing enzyme or metabolic pathway) to block the $NK^R \rightarrow NK^S$ shift that normally occurs in YAC populations that have not been treated with mutagen.

The basis of NK resistance has not been directly established but our hypothesis is that the alteration lies in an acceptor site (distinct from the NK-TS) for a putative lytic molecule released from the NK cell by intimate contact with the target, as previously suggested (3). The NK-TS itself was not appreciably altered; NK^R clones competed to an equal extent with NK^S clones in target competition assays. Furthermore, NK cells rec-

Table 3. Characteristics of NK^R YAC mutants

Parameter	YAC parent	YAC-19	YAC-6	YAC-6-28
Division time in logarithmic growth phase,[a] hr	16	18	24	26
Saturation density,[a] cells per 2-cm^2	6×10^5	5×10^5	1.2×10^5	1.0×10^5
Growth inhibition by wheat germ agglutinin,[b] I_{80}	75	100	56	80
Monoclonal anti H-$2K^k$,[c] 50% titer	1/512	1/512	1/1024	1/512
Monoclonal anti-thy 1,[d] 50% titer	1/2500	1/2500	1/2500	1/2500
Chromosome number[e]	41 and 39–42	41 and 39–42	41 and 39–41	41 and 39–42
DNA content in G_1 phase[f]	1.0	1.0	1.0	1.0
Erythrocyte/antibody rosettes,[g] %	0	0	0	0
Erythrocyte/antibody/complement rosettes,[h] %	0	0	0	0
Anti-Ly5.1,[i] 50% titer	1/512	1/1024	1/1024	1/1024
Normal rabbit serum,[j] 50% titer	1/32	1/32	1/32	1/32

[a] Cells were grown for 7 days at a starting concentration of 3.5×10^3/well in 1-ml 2.0-cm^2 wells (Linbro). Similar results were obtained in spinner flasks.

[b] Cells were grown from a starting concentration of 4×10^4/well for 5 days in the presence of various concentrations of wheat germ agglutinin, and the number of viable cells was determined. Data are expressed as the concentrationn of agglutinin (μg/ml) yielding 80% inhibition of growth. Other murine lines such as P815-2 or MDAY were inhibited by much less agglutinin (<10 μg/ml).

[c] Results represent the highest dilution of monoclonal anti-H-$2K^k$ yielding 50% lysis in the presence of rabbit complement.

[d] Dilution of monoclonal thy-1 (New England Nuclear) yielding 50% lysis in the presence of guinea pig complement (1:5).

[e] Results represent the modal number and range of chromosomes counted in 25 Giemsa-stained chromosome spreads for each cell line.

[f] Approximately 2×10^4 cells stained with propidium diiodide were analyzed by flow microfluoremetry. Results represent the relative DNA content of cells in G_1 phase of the cell cycle.

[g] Percentage of FcR^+ cells rosetting with sheep erythrocytes coated with IgG2b monoclonal anti-sheep erythrocyte antibody (1:100). Positive controls consisted of normal spleen cells and P388D macrophage tumor cells, which yielded 58% and 99% erythrocyte/antibody rosettes, respectively.

[h] Percentage of $C3R^+$ cells rosetting with sheep erythrocytes coated with subhemagglutinating amounts of rabbit IgM anti-sheep erythrocyte antibodies and mouse complement. Normal spleen and 2PK-3 Balb/C B-cell lymphoma cells yielded 40% and 95% erythrocyte/antibody/complement rosettes, respectively.

[i] Dilution of anti-Ly 5.1 antiserum yielding 50% lysis in the presence of rabbit complement.

[j] Cells were incubated for 1 hr at 4°C with heat-inactivated (56°C, 30 min) normal rabbit serum, washed, and incubated for 1 hr at 37°C with guinea pig complement (1:5). P815 cells were killed to a level of <10% at high concentrations (1:4) of normal rabbit serum.

ognized and bound to NK^R and NK^S cells to an equal extent as indicated by equal frequencies of target-binding cells. Finally normal rabbit serum, which has been shown to contain an antibody that may react with the NK-TS (10), reacted equally well with NK^R and NK^S cells. These results suggest that the NK resistance of the NK^R clones is not due to inadequate recognition or contact by NK cells.

We then asked whether NK^R simply reflected a membrane alteration rendering the cells generally more resistant to any form of lysis. It was found however, that cytolysis of NK^R cells was normal when mediated by (*i*) alloimmune T-killer cells educated *in vitro* or *in vivo*, (*ii*) lectin-dependent killer T cells activated *in vitro* or *in vivo*, or (*iii*) antibody and complement. Therefore the NK^R mutation does not reflect a general resistance to lysis but rather a selective resistance to NK cytolysis. This is not surprising; mutations at the effector level have been described in the postbinding cytolytic pathway that are selective for NK cells and not other effectors such as T cells or macrophages (14). Each effector cell type therefore must have a distinct cytolytic mechanism and possibly distinct classes of lytic molecules as well.

Other changes in the YAC cells have almost certainly occurred due to the mutagenic treatment by MNNG. For example, the cell division time and saturation density in the NK^R clones was lower than in the NK^S clone or the YAC parent. Minor differences were also observed in the ability of the cell lines to stimulate secondary mixed lymphocyte cultures *in vitro*. NK^R cells were better stimulators than NK^S cells (unpublished observation). The relationship, if any, of these cell properties to NK resistance is not known. In addition, it is not known whether or not NK resistance is under genomic control. Due to the relatively high frequency of NK^R variants obtained (10^{-1}), it is likely that NK resistance is under epigenetic control. It is instructive to consider the somatic cell genetics of NK resistance resulting from an absence of the NK-TS. When NK^S YAC cells were fused with a variety of NK-insensitive cell lines (e.g., A9HT), the hybrid was not NK^S and did not express NK-TS, whereas H-2 and viral antigens were codominantly expressed (15, 16). In hybrids between NK^R (P3Hr-1) and NK^S (K562) human cell lines, however (17), NK sensitivity in the hybrid was intermediate (15). It is not possible, therefore, to generalize concerning the genetic control of these "antigen-loss" variants or the postulated "acceptor-site" variants in the present study. Based solely on their high frequency of occurrence, however, we suggest that NK resistance due to acceptor-site alteration is analogous to certain differentiation states, such as immunoglobulin expression, that are readily suppressed. The acceptor site could simply be masked and indeed other workers have demonstrated a "counterlytic" mechanism in fibroblasts that is dependent on protein synthesis (18). In addition, we cannot exclude the possibility that NK^R clones may have some cytoplasmic alterations in metabolism that render them more resistant to NK-mediated cytolysis. Other workers, using repeated cycles of positive selection (i.e., NK killing) *in vitro* and *in vivo* have derived NK^R variants of YAC (19) and L5178Y (20) that lack the NK-TS for recognition. These antigen-loss variants represent a class of variants distinct from the putative Ag^+ acceptor-site variants described here.

In summary, we have characterized a partial, but selective, NK^R variant of YAC that should be useful in further delineating the cytolytic pathway of the NK cell. Two predictions can be made. The NK^R clones should exhibit greater tumorigenicity than NK^S clones in syngeneic NK-cell-bearing mice as demonstrated by Lavrovsky and Viksler with L cell variants (21). Finally, NK^R clones should be less suceptible to lysis by soluble "toxins" released from NK cells.

Note Added in Proof. Preliminary two-dimensional gel electrophoresis of YAC clone 19 and clone 6 shows a difference in at least one protein and that both of these cell lines differ from parental YAC.

We wish to express our gratitude for the excellent technical assistance of Mses. M. Florian, R. Page, and M. S. Mann, and we thank Miss Dianne Robertson for typing this manuscript. This work was supported by the Medical Research Council and the National Cancer Institute of Canada. R.S.K. is a Research Scholar and D.K. is a Research Student of the National Cancer Institute of Canada. T.H. is a Fellow of the Medical Research Council of Canada.

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