TRANSFECTION OF A RAT CELL LINE WITH THE v-Ki-ras ONCOGENE IS ASSOCIATED WITH ENHANCED SUSCEPTIBILITY TO NATURAL KILLER CELL LYSIS

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Natural killer (NK) cells have been proposed as a first level of defense against the initiation and spread of tumors in vivo (1). Recent studies with syngeneic mice have directly correlated levels of NK activity with resistance to tumor growth (reviewed in 2). However, most of these investigations have used long-passaged transplantable tumor cell lines rather than newly transformed cells. We had predicted that, if NK cells played a true surveillance role, host cells should become susceptible to NK-mediated cytotoxicity during transformation to the malignant phenotype. The present study demonstrates that NK-resistant rat fibroblasts transformed with the v-Ki-ras oncogene become anchorage independent, grow as tumors in nude mice, and acquire NK sensitivity. These results indicate that NK cells can recognize and kill newly transformed cells and that conceivably they play an early role in host surveillance against neoplasia.

Materials and Methods

Cells. Rat-1, a cell line derived from Fisher rat F2408 fibroblasts (3), was transfected and injected into NIH-2 nu/nu mice (2 × 10⁶ cells per mouse, subcutaneously). Large growing tumors were explanted in vitro after 3 wk and reestablished as cell lines. Injection of nontransfected rat-1 cells resulted in a barely palpable, benign nodule, which was also established as a cell line and used as a control in most experiments. The NK clones LH 49 (cytolytic) and L250-A9 (less cytolytic) were gifts of Dr. C. Brooks (4).

Transfection Protocol. Rat-1 was transfected with the entire 7.0 kilobase (kb) v-Ki-ras oncogene ligated into the Eco RI site of pBR322 (5). The transfection procedure has been previously described (6). Transfected cells were selected by colony formation in 1% methyl cellulose at 3–6 wk (7).

Cytotoxicity Assays. Target cells labeled with ⁵¹Cr were analyzed in 6-h assays with a variety of effectors (see Table II) as previously described (1).

Southern Blot Analysis of Transfectants. DNA (15 μg) was digested with Eco RI, blotted (8), and hybridized to a 0.6 kb Kpn I fragment of v-Ki-ras corresponding to the long-terminal repeat sequence of the oncogene. After washing under high stringency, autoradiograms were exposed overnight.

Results and Discussion

Cytotoxicity assays performed on transformed and untransformed lines demonstrated that transfection of the v-Ki-ras oncogene enhanced NK-mediated...
killing. As shown in Fig. 1, the transformed cells were much more sensitive \((P < 0.01)\) than the rat-1 parent to lysis by nylon wool-passed spleen cells from poly(I)C-boosted CBA/J mice. This difference was \(>20\)-fold in terms of lytic units calculated at 10% lysis. Since the parent cells are essentially resistant to NK lysis, they required \(>10^6\) lymphocytes to even approach this level of killing. The difference was even more striking when the LH49 NK clone was used as an effector.

Transformed cells injected subcutaneously into nude mice grew as tumors, and were subsequently reestablished in culture. Cytotoxicity assays performed on tumor cells passaged through nude mice consistently showed that they were also highly NK sensitive. As summarized in Table I, data from seven replicate experiments demonstrate that the newly transformed and tumor-derived Ki-ras-A cells are \(26-58\)-fold more NK sensitive than rat-1, and \(6-12\)-fold less sensitive than the standard NK-sensitive tumor, YAC 1.2. Both the parental rat-1 cells and a line established from a benign, barely palpable nodule at the site of injection of untransformed rat-1 cells were NK resistant (\(<1\) lytic unit per \(10^6\)). Two additional, independently derived rat-1 transformants (Ki-ras-B and -C) were also converted to NK sensitivity after transfection with v-Ki-ras (Table I).

![Figure 1](image-url)
**Table I**

NK Sensitivity in a Panel of Transfected Targets and Nontransfected Controls

| Target cells          | No. of experiments | Cytolysis       |
|-----------------------|--------------------|-----------------|
|                       |                    | LU/10⁶          | Percent lysis (100:1 E/T) |
| YAC 1.2               | 7                  | 392 ± 122       | 42 ± 2                    |
| Rat-1                 | 5                  | <0.5 ± 0.2      | 3 ± 2                     |
| Nodole                | 6                  | 1 ± 0.9         | 7 ± 2                     |
| K-ras A               | 7                  | 26 ± 12         | 23 ± 10                   |
| K-ras A tumor         | 7                  | 38 ± 20         | 32 ± 6                    |
| K-ras B               | 2                  | 6 ± 1           | 20 ± 4                    |
| K-ras B tumor         | 1                  | 10              | 17                        |
| K-ras C               | 2                  | 8 ± 2           | 18 ± 2                    |
| K-ras C tumor         | 4                  | 11 ± 5          | 21 ± 5                    |

Fresh spleen cells from CBA/J mice boosted 24 h previously with 100 μg of poly(I)C were titrated in serial dilutions at effector/target (E/T) ratios of between 200:1 and 3:1. Lytic units (LU) were calculated from titration curves; and 1 LU is defined as the number of lymphocytes required to lyse 10% of 51Cr-labeled targets in a 6-h assay; the values shown represent LU per 10⁶ effector lymphocytes. Percent lysis is indicated at the 100:1 effector/target ratio. Data represent the mean and standard error of replicate experiments.

**Table II**

Characterization of the NK Effector Cell

| Strain       | Assay time | Poly (IJC) | Spleen fractionation | Treatment | YAC Lysis% | YAC Lysis LU/10⁶ | Ki-ras A tumor Lysis% | Ki-ras A tumor LU/10⁶ |
|--------------|------------|------------|----------------------|-----------|------------|-----------------|-----------------------|------------------------|
| CBA/J        | 6 –        | None       | None                 | Poly (IJC) | 33         | ND              | 11 ± 8               | 8 ± 1                  |
|              | 6 -        | None       | None                 | Poly (IJC) | 42         | ND              | 26 ± 5               | 50 ± 1                 |
|              | 10 -       | Nylon wool | Anti-Lyt-1.1, 2.1    | Poly (IJC) | 80         | 160 ± 54       | 55 ± 45              | 45 ± 25                |
|              | 10 +       | Nylon wool | Anti-Lyt-1, 2.1      | Poly (IJC) | 82         | 250 ± 52       | 52 ± 50              | 50 ± 50                |
|              | 10 +       | Nylon wool | Anti-Lyt-1.1, 2.1, asialo GM ** | Poly (IJC) | 63         | 125 ± 28       | 28 ± 25              | 25 ± 25                |
| BALB/c       | 10 +       | Nylon wool | Anti-Lyt-1, 2.1      | Poly (IJC) | 74         | 100 ± 55       | 35 ± 41              | 41 ± 35                |
|              | 10 +       | Nylon wool | Anti-Lyt-1, 2.1, NK-2A | Poly (IJC) | 44         | 34 ± 1        | 1 ± 1               | <1 ± 1                 |
|              | 10 +       | Nylon wool | Anti-Lyt-1, 2.1, NK-2A | Poly (IJC) | 47         | 31 ± 2        | 2 ± 1               | <1 ± 1                 |
| DBA/2        | 4 +        | Nylon wool | None                 | Poly (IJC) | 17         | 18 ± 12       | 12 ± 10              | 10 ± 10                |
| Wistar-Firth | 4 +        | Nylon wool | None                 | Poly (IJC) | 17         | 25 ± 13       | 53 ± 115             | 115 ± 115              |
| C57Bl/6      | 6 –        | —          | NK clone LH 49       | No poly (IJC) | 21         | 500 ± 41       | 41 ± 1000            | 1000 ± 1000            |
|              | 6 –        | —          | NK clone L250-A9     | No poly (IJC) | 7          | 62 ± 9        | 9 ± 110              | 110 ± 110              |

* Percent cytosis of 51Cr-labeled target cells at a 100:1 effector/target ratio.

† A lytic unit (LU) was defined as the number of lymphocytes required to lyse 10% of targets. The value shown represent LU per 10⁶ effector lymphocytes.

‡ Mice were boosted intraperitoneally with 100 μg (1 U) of poly(IJC) 24 h before assay.

§ Spleen cells were pooled from 4 to 18 animals per group and passed over nylon wool columns.

** 10⁷ Spleen cells/ml were treated 1 h at 4°C with a 1:20 dilution of monoclonal anti-Lyt-1.1 mixed with 1:20 anti-Lyt-2.1 (Cedarlane Laboratories), followed by washing and a 1-h incubation at 37°C with 1:10 rabbit complement preabsorbed with mouse tissues.

*** After anti-Lyt-1.1, 2.1 and complement treatment, spleen cells were treated with 1:100 rabbit anti-asialo GM1 sera or 1:80 mouse anti-NK-1.2 or -2.1 sera followed by 1:10 rabbit complement. These experiments were repeated 3 times with similar results.

whereas cells transfected with v-fes remained NK resistant (data not shown).

The characteristics of effector cells that caused cytosis of transfected targets are summarized in Table II. Although normal, unfractionated CBA/J mouse spleen cells killed the Ki-ras tumor, boosting of the mice with poly(IJC) resulted...
in a sixfold enhancement of the effect. The poly(I)C augmentation was less apparent when using nylon wool column-passed spleen cells depleted of Lyt-1+ and Lyt-2+ effectors. In addition to being Lyt-1-,2- phenotype, the antitumor effector cells were partially depleted by treatment with anti-asialo GM1, and totally eliminated by treatment with anti-NK-1.2 or NK-2.1. Experiments with cloned NK cell lines of varying cytolytic potential, kindly provided by C. Brooks (4), demonstrated that the clones lysed YAC-1.2 and the transfected tumor line equally, but had negligible effects on the rat-1 parent. The phenotype of the effector cell that mediates cytolysis of rat-1 transformants is Lyt-1-,2-, NK-1.2+, NK-2.1+, asialo GM1 positive, nylon wool nonadherent, and poly(I)C boostable. Therefore, the effect is most likely mediated by NK cells.

The nature of the presumptive target structures on the transformants was investigated using cold-target competition studies to ascertain if determinants were different from those expressed on YAC 1.2. As shown in Table III, unlabeled Ki-ras A tumor cells competed with NK cells for lysis of 51Cr-labeled YAC. The degree of inhibition was comparable to that observed between labeled and unlabeled YAC. Similar results were obtained in the reciprocal experiment in which the Ki-ras A tumor cells were labeled and killing was reduced by competition with either unlabeled Ki-ras-A tumor cells or unlabeled YAC 1.2. Nontransformed cells did not compete in either system.

Transfection was verified by Southern blot analysis of genomic DNA hybridized to a 0.6 kb segment from the original v-Ki-ras-transfected gene (Fig. 2). This fragment, which corresponds to the viral long-terminal repeats (LTR) in the oncogene, hybridized strongly to DNA from the transfected cells but not control lines, under the conditions described above.

These results suggest that transformation of NK-resistant fibroblast lines with specific oncogenes such as v-Ki-ras converts the cells to an NK-sensitive, anchorage-independent, malignant phenotype. Tumor progression is likely related to a loss of NK sensitivity since studies of rat fibroblast cell lines transformed with adenovirus 2 have revealed an inverse correlation between NK susceptibility and

### Table III

| Labeled target       | Unlabeled competitor | Competitors (×10⁵) required for 50% inhibition of lysis |
|----------------------|----------------------|-------------------------------------------------------|
| YAC 1.2              | YAC 1.2              | 8                                                     |
|                      | K-ras A tumor        | 4                                                     |
|                      | Rat-1                | >64                                                   |
|                      | Nodule               | >64                                                   |
| K-ras A tumor        | YAC 1.2              | 4                                                     |
|                      | K-ras A tumor        | 4                                                     |
|                      | Rat-1                | >64                                                   |
|                      | Nodule               | >64                                                   |

YAC 1.2 and nude mouse-passaged, v-Ki-ras A-transfected, rat-1 tumor cells were labeled with 51Cr and tested in a 6-h cytotoxicity assay against nylon wool-passed spleen cells from six CBA/J mice injected 24 h previously with 100 µg (1 U) of poly(I)C acid. Varying numbers of unlabeled target cells were added to a constant number of 51Cr-labeled targets (2 × 10⁵/well) at an effector/target ratio of 100:1. The mean percent lys values from triplicate wells were plotted and the number of competitors causing 50% inhibition of cytolysis was calculated by interpolation. This experiment was repeated five times with similar results.
FIGURE 2. Southern blot of v-Ki-ras A transfectants. DNA was extracted from transfected and untransfected cell lines and equal amounts were digested with Eco RI, electrophoresed on an agarose gel, and transferred to Gene Screen Plus by Southern blotting. Blots were probed with a 0.6 kb fragment of the transfected gene and autoradiographed. Tracks correspond to equal amounts of DNA from (A) rat-1; (B) benign nodule at the site of injection of rat-1 into nude mice; (C) v-Ki-ras A transfectants; (D) tumor from nude mice injected with v-Ki-ras A transfectants. Size markers in kilobasepairs are shown on the left.

The results indicate that the NK surveillance system can recognize and kill cells newly transformed by a member of the ras oncogene family.

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