

TARGET-EFFECTOR INTERACTION IN THE
HUMAN AND MURINE NATURAL KILLER SYSTEM
Specificity and Xenogeneic Reactivity of the Solubilized Natural Killer-
Target Structure Complex and Its Loss in a Somatic Cell Hybrid*

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Natural killer (NK)¹ cells are unique in the lymphoid system because there is no H-2 restriction of killing (1-3), no classical immunological memory, and these cells kill a wide range of unrelated target cells of both tumor (4) and nontumor origin (5). More importantly, however, NK cells pre-exist at high levels in the host (6) in contrast to other effector mechanisms (T cells, antibody-dependent cell-mediated cytotoxicity and macrophages) which require days or weeks to be primed. NK cells, therefore, might be expected to provide a first line of defence against newly arising malignancies and it was important to investigate the specificity of the target-effector interaction in this system because little is known regarding the nature of the recognition structure or the target sites involved. It is possible that the receptors are coded by the immunoglobulin variable region genes or perhaps they represent remnants of a more primitive surveillance mechanism.

Previous work has shown that various unlabeled competitor cells could inhibit isotope release from labeled target cells in a cytolytic assay and this observation was used to infer that the target and competitor shared common determinants which were recognized by surface receptors on the NK cell (1, 2). To directly visualize target-effector recognition and binding, we have devised a target binding cell (TBC) assay which detects individual NK cells (6, 7). The recognition receptor on the NK cell appears to be coded for by genes linked to the H-2 complex (6).

In a previous report we showed that preincubation of effector cells with glycoproteins isolated from the surface of various tumor cells, selectively bound to NK cells and specifically inhibited their attachment to the homologous intact target cell (8). These molecules, designated natural killer cell target structure (NK-TS), were not detected on NK-insensitive targets and had no effect on alloimmune T-cell binding to H-2 specific targets. To extend the NK-specificity studies, we have isolated NK-TS from a number of divergent tumor cell lines and performed extensive cross-inhibition

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¹ *Abbreviations used in this paper:* BSS, balanced salt solution; FITC, fluorescein isothiocyanate; NK, natural killer cell; NK-TS, natural killer cell target structure; SDS, sodium dodecyl sulfate; TBC, target binding cell.

studies. In addition, some factors which regulate the expression of NK-TS and its recognition by NK cells are reported here.

Materials and Methods

Mice. 6- to 10-wk-old mice were used in all experiments and various strains were maintained in this laboratory by continuous single-line, brother-sister mating.

Tumor Cell Lines. Unless stated otherwise, all tumor cells were maintained by continuous in vitro culture and originated as follows. YAC, Moloney leukemia-induced lymphomas of A/Sn; P815, methylcholanthrene-induced mastocytoma of DBA/2 mice; X-63, mineral oil-induced plasmacytoma of BALB/c mice; YAC-IR, a subline of YAC (9); A9HT, an L-cell-derived line; YAC-IR/A9HT, an in vitro fusion product (10); Molt-4, human T-cell line from acute lymphatic leukemia patient; and K562, human myeloid leukemia line from chronic myeloid leukemia patient.

Nylon Wool Columns. Monodispersed cells from various lymphoid organs were treated briefly (4 s) with H₂O to remove erythrocytes by hypotonic shock and the remaining cells were passed over nylon wool columns with cell recoveries between 10 and 20% of input.

Preparation of Human Fc Receptor Positive Lymphocytes. Fc receptor positive human lymphocytes were isolated by protein A elution of cells bound to immune complexes insolubilized on plastic Petri dishes by the MIC (monolayer-immune complex) procedure (11). Briefly, Falcon Petri dishes (Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.) were first treated with purified IgG (5 mg/ml), washed, and then treated with rabbit anti-IgG. Peripheral blood lymphocytes were then gently spun onto the dishes and nonattached cells were poured off. To elute the remaining, complex-bound Fc receptor-positive lymphocytes, the plates were slowly rocked for 2 h in the presence of protein A (30 µg/ml) in phosphate-buffered saline (PBS) with 10% fetal calf serum. Before use, the eluted cells were washed three times.

Target Binding Cell Assay. Lymphoid cells, depleted of erythrocytes by hypotonic shock, were labeled with fluorescein isothiocyanate (FITC, BDH Chemicals Ltd., Poole, England) as previously described (7). 10⁶ YAC cells were then mixed with 10⁵ FITC-labeled lymphoid cells in 0.1 ml RPMI (Grand Island Biological Co., Grand Island, N. Y.) plus 10% fetal calf serum and centrifuged at 200 g for 5 min at room temperature in round-bottomed microtiter plates. The plates were placed on ice for 30 min and the cells were then aspirated 5–10 times with a Pasteur pipette. One drop of cell suspension was placed on a microscope slide and counted under a UV microscope. The percentage of fluorescing cells (effectors) binding to nonfluorescing cells (targets) was then determined after counting 300–400 effector cells. For inhibition studies, gel extracts were preincubated with spleen cells in balanced salt solution for 15 min at room temperature followed by 45 min on ice before mixing with target cells. Variation between replicate samples was always <10%.

Detergent Extraction of Cells. Tumor cells were resuspended at a concentration of 2 × 10⁸/ml in extraction medium containing 4 mM Triton X-100 (0.25%) (Packard Instrument Co., Inc., Downer's Grove, Ill.) 1 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 0.02% sodium azide as described by Troy et al. (12). Cells were incubated at 37°C for 2 h and centrifuged at 48,000 g for 1 h at 4°C. Supernates were then prepared for gel electrophoresis.

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis. The Triton X-100 solubilised and reduced proteins were added to starting buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycine, and 62.5 mM Tris buffer, pH 6.8) and heated for 10 min at 85–90°C in a boiling water bath. Slab gel electrophoresis was carried out in 0.1% SDS as a modification (12) of the method of Laemmli (13).

After electrophoresis, gel slabs were washed in balanced salt solution (BSS) for 24 h as described by Troy et al. (12) under conditions which removed most of the detergent but little if any protein. Channels containing cell extracts or molecular weight standards were cut vertically and stained for protein with Coomassie Brilliant Blue R-250. Additional channels containing cell extract were sliced horizontally in 2 to 5-mm sections as shown in Fig. 1. Gel slices were added to small tubes and macerated in the presence of 0.15 ml buffer. Tubes were incubated 24 h at 4°C and the supernates were then used as a source of antigenic material.

The following purified proteins were used as molecular weight markers: unreduced ferritin

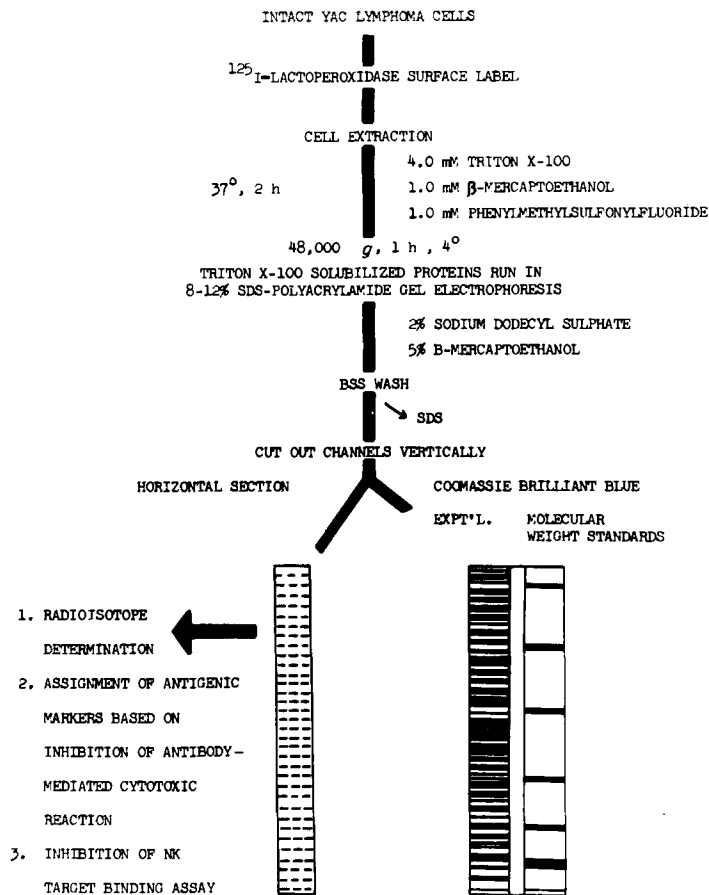


FIG. 1. Schematic diagram of experimental procedures. Intact YAC cells were treated with Triton X-100 with or without prior surface labeling with ^{125}I using the lactoperoxidase method. Solubilized components were electrophoresed in SDS-polyacrylamide gels which were subsequently stained for protein with Coomassie Blue. Additionally, parallel channels were then cut in horizontal into 2 to 5-mm slices and macerated in BSS. Aliquots from each gel fraction were removed after at least 24 h at 4°C and assayed for various antigenic markers by inhibition of an antibody-mediated microcytotoxicity assay. Additional aliquots were tested for inhibition of NK cell binding to intact target cells.

(480K), IgG nonreduced (150K), bovine serum albumin (68K), reduced catalase (60K), ovalbumin (45K), reduced aldolase (40K), chymotrypsin A (25K), horse myoglobin (17.8K), and reduced cytochrome C (12.4K). The SDS-protein ratio used in all these experiments was 4:1.

Inhibition of Antiserum-Mediated Cytotoxicity. YAC cells were used as targets in a microassay as previously described (14). Antiserum from (ASW \times C57BL/6)F₁ anti-CBA was used to detect H-2D^kK^k in gels of A9HT cells. CBA anti-DBA serum was used to detect H-2D^d in gels of YAC-IR and YAC-IR/A9HT hybrids. Monospecific goat anti-Friend virus gp71 serum was kindly provided by Dr. W. Shafer (Max-Planck-Institute fur Virus-forschung, Tubingen). This anti-viral sera did not react with normal lymphocytes or control cell lines and was completely inhibited by the corresponding purified protein (15).

6- to 10-fold and 3- to 5-fold serial dilutions of gel supernatant material was added to antiserum dilutions that gave 60-90% killing. Antigenic units are expressed as the reciprocal dilution corresponding to 20% inhibition.

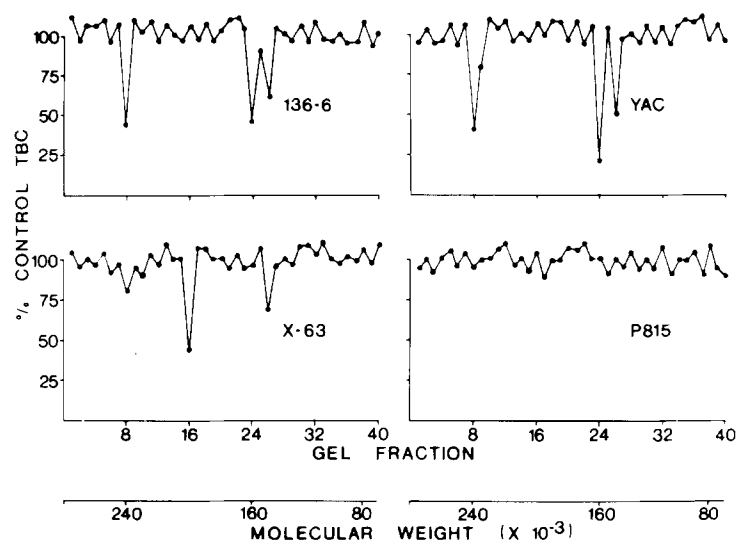


FIG. 2. The isolation of NK-target structures (NK-TS) from four distinct murine cell lines. Identical quantities of Triton X-100 solubilized and reduced proteins (4 mg/ml) from YAC (Moloney virus-induced lymphoma), 136-6 (Radiation leukaemia virus induced lymphoma), X-63 (plasmacytoma), or P815 (mastocytoma) were electrophoresed in parallel channels in a 5-7% SDS-polyacrylamide gradient. Proteins were removed from individual gel slices and assayed for inhibition of binding by nylon-passed CBA spleen cells to intact homologous YAC, 136-6, or X-63 targets. Because P815 is NK insensitive, this gel was assayed for inhibition of binding to YAC. The gel region between 70K and 350K is shown because all other fractions failed to inhibit the TBC assay. The frequency of TBC in controls was 23%, YAC; 10%, X-63; 8%, 136-6; 1%, P815. Immediately after growing up large quantities of tumor cells for extraction, cytotoxicity by nylon-passed CBA spleen cells at a 50:1 effector:target ratio in a 4-h cytolytic assay was 62%, YAC; 26%, X-63; 16%, 136-6; and 2%, P815.

Results

A Comparison of NK-TS Isolated from Murine Tumors. The NK-TS profiles of three NK-sensitive and one NK-insensitive mouse cell lines are shown in Fig. 2. Detergent extracts of YAC, 136-6, X-63, and P815 cells were electrophoresed in parallel channels in SDS-polyacrylamide gels and proteins eluted from individual gel slices were preincubated with CBA spleen cells and assayed for inhibition of NK attachment to the homologous intact target. Inhibitory material was designated NK-TS because we have previously shown that it contains plasma membrane-derived glycoproteins which selectively bind to putative recognition receptors on NK cells and specifically inhibit TBC (8). As shown, the reduced NK-TS from YAC, a T-cell lymphoma, migrated as three molecular species with apparent M_r values of 140K, 160K, and 240K which are within $\pm 10K$ of values calculated earlier (8). Another T-cell lymphoma, 136-6, exhibited a profile identical to that of YAC, whereas X-63, a plasmacytoma, expressed only the 140K peak in common. The 160K and 240K peaks were not detectable and a new peak appeared in the molecular weight region between 190 and 200K. The protein bands corresponding to the inhibitory fractions are shown in Fig. 3 and are clearly visible in YAC and 136-6 with the exception of the fraction 8 band which is rather broad and diffuse. X-63 lacked a band in the position of fraction 8 and 24 and exhibited a new band at fraction 16 as expected. P815, an NK-insensitive line,

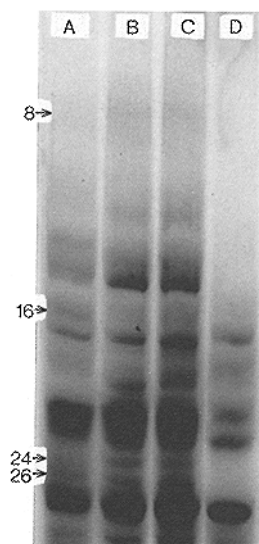


FIG. 3. SDS polyacrylamide gel electrophoresis profiles of Triton X-100 solubilized and reduced proteins from various cell lines. Gels were stained with Coomassie Blue and the regions shown correspond to Fig. 2. A, X-63; B, 136-6; C, YAC; and D, P815.

exhibited no NK-TS peaks (Fig. 2) and also lacked all of the corresponding protein bands in fractions 8, 16, 24, and 26 (Fig. 3). Therefore, most, if not all, of the protein in these bands is comprised of NK-TS. In further experiments not reported here, each NK-TS band exhibited very little microheterogeneity (one band) upon isoelectric focussing and had an isoelectric point of pH 4.2. In additional experiments, a 20K disulphide-linked side chain was detected in at least one of the NK-TS molecules (J. Roder, unpublished observation).

As shown in three-way cross-inhibition experiments, the 140K peak of NK-TS cross-reacted between YAC, 136-6, and X-63, whereas the 160K and 240K peaks bore unique specificities (Fig. 4). In X-63, the 140K peak cross-reacted with YAC and 136-6, whereas the 190K peak was unique. The small amount of inhibition in fraction 8 on the X-63 gel was not consistently observed.

Xenoreactivity of NK-TS Isolated from Human Tumor Cells. The NK-TS profiles of Molt-4 and K562 are shown in Fig. 5. When gel eluates were assayed for inhibition of TBC formation by human peripheral blood lymphocytes enriched for FcR-bearing cells, three characteristic peaks were observed in the molecular region of 140K, 160K, and 240K. When the same gels were screened using mouse spleen cells as effectors, a different pattern was obtained. Mouse spleen cells were only inhibited by the 140K peak from K562 or the 140K and 160K peaks from Molt-4. Although Molt-4 and K562 are sensitive to NK lysis by both human and mouse effectors, it is apparent that each species recognises a different set of surface molecules on these targets.

In cross-inhibition experiments using human effectors, the 240K peak of NK-TS from Molt-4 bore distinct determinants compared to K562, whereas the two lower molecular weight peaks cross-reacted (Fig. 6).

Loss of NK-TS on a Somatic Cell Hybrid between NK Sensitive and Insensitive Cell Lines. As shown in Table I, YAC-IR was highly sensitive to NK-mediated binding and lysis by

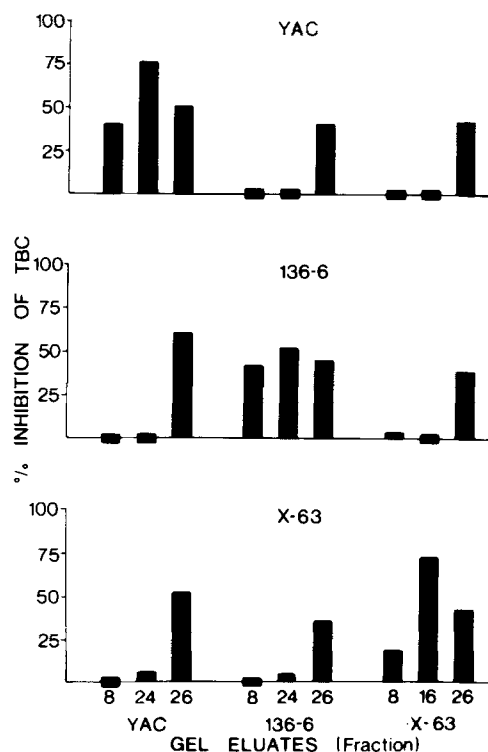


FIG. 4. Cross-inhibition of TBC with NK-TS from various murine cell lines. For each cell line, the NK-TS peak was pooled from several gels such as the one shown in Fig. 2. The protein in each NK-TS peak for each cell line was equalized to $10 \mu\text{g/ml}$. This material was then preincubated with nylon-passed CBA spleen cells and tested for inhibition of TBC to the corresponding intact target or to the other heterologous targets. The data is expressed as the mean percent inhibition of TBC from triplicate determinations. All inhibition $>20\%$ was significant at the $0.001 < P < 0.02$ level.

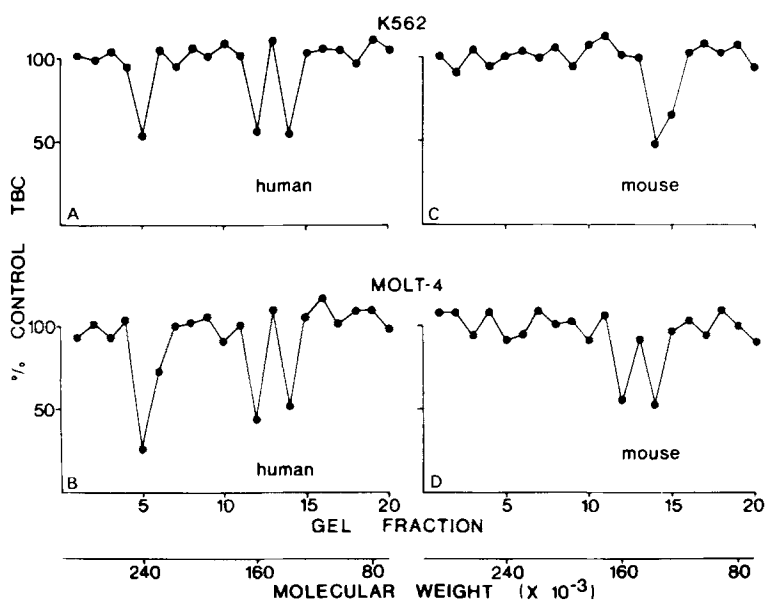


FIG. 5. The isolation of human NK-TS. Triton X-100 solubilized and reduced proteins from Molt-4 or K562 cells were electrophoresed in SDS-polyacrylamide gels. Proteins were removed from individual gel slices and incubated with FcR^+ , human peripheral blood lymphocytes to assay for inhibition of binding to the corresponding intact target (A, B). Aliquots from each fraction were also preincubated with mouse (CBA) spleen cells (nylon passed) for inhibition of TBC (C, D).

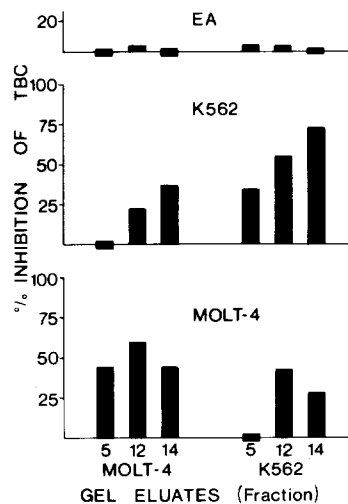


FIG. 6. Cross-inhibition of TBC with NK-TS from Molt-4 and K562. NK-TS were pooled from several gels such as the one shown in Fig. 5. Protein in each fraction for each cell line was equalized to 10 $\mu\text{g/ml}$ and then tested for inhibition of human effector cell (FcR^+ -selected peripheral blood lymphocytes) binding to Molt-4, K562, or antibody-coated ox erythrocytes. Control levels of TBC were 15% EA; 30% K562, and 22% Molt-4.

TABLE 1
The Loss of NK-Cell Binding in YACIR/A9HT Compared to Its Parental Cell Lines

| Cell line | NK lysis* | | NK TBC‡ | |
|----------------------------------|-----------|--|---------|--|
| | % | | % | |
| YAC-IR (H-2 ^a) | 41 | | 21 | |
| A9HT (H-2 ^b) | 3 | | 5 | |
| YACIR/A9HT (H-2 ^{a+b}) | 4 | | 6 | |

* Effector/target cell ratio 50:1 in a 4-h ⁵¹Cr-release assay. Similar results were obtained with a 25:1 ratio. CBA spleen cells were used as effector cells.

‡ Nylon wool-passed CBA spleen cells were fluorescein labeled and incubated with a 10-fold excess of target cells.

CBA effectors, whereas A9HT was virtually insensitive. The in vitro fusion product of these two parental lines, YAC-IR/A9HT, exhibited low NK activity in both the TBC and cytolytic assays. This data confirms previous NK cytolytic experiments on these cell lines (16).

In addition, the NK-TS could not be detected in SDS-polyacrylamide gels run with solubilised and reduced extracts of A9HT or the A9HT/YAC-IR hybrid. YAC-IR exhibited three characteristic peaks of NK-TS and all three cell lines expressed serologically detectable gp71 and the appropriate H-2 antigens in the expected molecular weight regions (Fig. 7). This is in line with cytotoxic results on this hybrid (16).

Discussion

These results show that NK-TS that are isolated from tumor cells of widely differing histogenic origin, exhibit striking similarities in physical-chemical properties. Although unique specificities are carried by some of the multiple NK-TS protein molecules, cross-reactions are widespread.

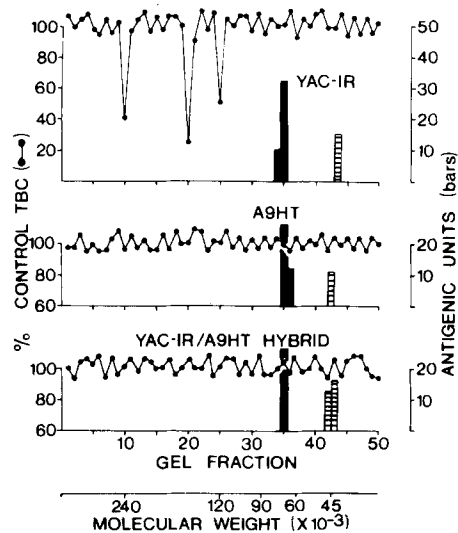


FIG. 7. The location of NK-TS in SDS-polyacrylamide gels. Identical amounts of Triton X-100 solubilized and reduced proteins (4 mg/ml) from YAC-IR, A9HT, or YAC-IR/A9HT hybrid cells were electrophoresed in 8% SDS-polyacrylamide gels. Proteins were removed from individual gel slides and all fractions were assayed for antigenic activity by the inhibition of antiserum-mediated cytotoxicity (bars) or by inhibiting the binding of NK cells to intact YAC-IR targets (closed circles). The frequency of nylon-passed CBA spleen cells binding to YAC-IR cells (TBC) in controls was 25%. The H-2 antigens assayed included H-2D^d for YAC-IR; H-2K^kD^k for A9HT, and H-2K^dD^d for the YAC-IR/A9HT hybrid. Symbols: ●, % control TBC; ▨, gp71; ■, H-2.

Tumor cells of T-cell (YAC, 136-6) or B-cell (X-63) origin in the mouse share NK-TS specificities as revealed in cross-inhibition tests (Fig. 4). Similarly, HLA-negative myeloid cells (K562) of human origin share NK-TS specificities with a T-cell lymphatic leukemia (Molt-4, Fig. 6). When the three NK-TS molecules were compared in a number of different cell lines, the large 240K molecules most often carried the unique NK specificity, whereas the smaller 140K molecule always cross-reacted in the combinations tested. The 160K molecule may have either property depending on its origin in mice or humans. The identity of the NK-TS has not yet been associated with any known cell surface protein but it is clearly distinct from H-2, gp71, p30, p15E, or Moloney cell surface antigen in YAC cells (8). The widespread nature of common NK-TS might suggest that the NK-TS are (a) derepressed fetal antigens of a highly conserved nature, (b) differentiation antigens, or (c) environmentally determined antigens. Although it is not possible to distinguish between these alternatives at present, it is interesting to note that normal unimmunised rabbits have naturally occurring IgG in their serum with similar specificity as mouse NK cells which suggests that an infectious agent common to rabbit and mouse may generate antibody in the rabbit and NK cells in the mouse (17). It has also been shown that immature cells in the normal thymus are sensitive to lysis by mouse NK cells and rabbit natural antibodies (17).² Therefore, the NK-TS may not be directly dependent on neoplastic transformation for expression.

² Hansson, M., K. Karre, R. Kiessling, J. C. Roder, B. Andersson, and P. Hayry. 1979. Natural NK-cell targets in the mouse thymus; characteristics of the sensitive cell population. *J. Immunol.* In Press.

The main bioassay for target structures in the present study involves competitive inhibition of target cell binding by effector cells. Effectors are preincubated with various solubilized target cell fractions and these effectors are then used in binding assays with intact target cells. It is assumed that inhibition of binding over controls indicates that target molecules have bound to recognition structures on the effector cell, thereby preventing effective interaction with the intact targets. These assumptions have been checked by showing that radiolabeled target cell antigens are absorbed by NK cells, thereby eliminating the possibility that inhibition of the effector resulted from a nonspecific enzymatic-like activity in the various fractions (8). Furthermore, the absorption is specific because (a) radiolabeled surface proteins from NK-insensitive targets are not absorbed by NK cells, and (b) the quantity of antigen absorbed from sensitive targets varied directly with the concentration of NK cells in the absorbing population.³ As discussed elsewhere, it is valid to use the NK target cell binding assay as an indicator system because it has been conclusively shown that target binding cells in our assay represent NK cells (6, 7).

Factors which may be important in the expression of NK-TS were also examined. A somatic cell hybrid between NK sensitive and NK insensitive parental cell lines did not express the NK-TS (Fig. 7) and did not give positive results in the TBC assay (Table 1) which is in accordance with negative results in the NK cytolytic assay as previously reported (16). Several additional hybrids in NK high/low combinations were also low in NK sensitivity (16). These results suggest that low NK sensitivity in the hybrids is not simply a result of masking of the NK-TS. It is not yet known, however, if the loss of NK-TS is a result of a selective loss of chromosomes coding for NK-TS or rather a suppression of the NK-TS gene(s) as occurs with certain differentiation markers (16).

A different form of NK-TS modulation was also shown to occur (8). YAC cells, when removed from the selective pressures of an NK-containing milieu *in vivo* and grown *in vitro*, gradually exhibit increasing sensitivity to NK-mediated lysis over a period of weeks (18). We have previously shown a concomitant increase in the quantitative expression of NK-TS in these *in vitro* explanted cells (8). Cell cycle effects may not be important in the expression of NK-TS because NK cells bind to targets equally well which are at various stages in the cell cycle (J. Roder, unpublished observation).

The available evidence supports the concept of limited heterogeneity in the putative NK recognition receptor. Hence NK cells from high (CBA) and low (A/Sn) NK reactive strains exhibited identical avidities for NK-TS from YAC cells.² That some heterogeneity in the receptor exists is suggested by the observation that receptor avidity increases in early postnatal development in CBA mice.² In addition, NK cells from mice recognised different patterns of NK-TS than did human NK cells (Fig. 6). Because several unique NK-TS specificities have also been found (Figs. 4, 6) then these observations, taken together, suggest that the NK cell pool is poly-specific and has some heterogeneity in the recognition structure, albeit much less than would be expected of an antibody-combining site. The clonal distribution of these various NK receptors is now amenable to investigation.

³ Roder, J. C. 1979. Target-effector interaction in the natural killer cell system. VI. The molecular basis of age-dependent and genetic variation in NK function. Submitted for publication.

Summary

Preincubation of natural killer (NK) cells with electrophoresis purified proteins from a variety of NK-sensitive murine and human tumor cells specifically prevented subsequent binding to the intact, homologous target cell. The NK-target structures (NK-TS) consisted of some or all of four characteristic molecular species, tentatively assigned molecular weights of 140K, 160K, 190K, and 240K (± 10 K) based on electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gels. When these NK-TS molecules were compared in cross-inhibition assays, the large 240K molecule most often carried the unique NK specificity, whereas the smaller 140K molecules cross-reacted between YAC, 136-6 and X-63 in the mouse and between Molt-4 and K562 in the human. Mouse NK cells recognised a different spectrum of NK-TS molecules than human NK cells. The control of NK-TS expression was partially revealed in a cloned, somatic cell hybrid between an NK sensitive (YAC-IR) and insensitive (A9HT) cell line. The hybrid did not express NK-TS and did not bind to NK cells which is in accordance with negative NK cytolytic results previously reported.

Although unique specificities are carried by some of the multiple NK-TS protein molecules, cross-reactions were widespread. These observations taken together suggest that the NK cell is polyspecific and has some heterogeneity in the recognition structure although much less than would be expected of an antibody-combining site.

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