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Target–effector interaction in the natural killer (NK) cell system

VI. THE INFLUENCE OF AGE AND GENOTYPE ON NK BINDING CHARACTERISTICS

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Summary. Four independent assays were used to compare target-cell binding by NK cells in different populations. First, detergent solubilized and reduced proteins from the surface of Moloney lymphoma cells (YAC) were electrophoresed in SDS-polyacrylamide gels. The glycoproteins recognized by NK cells (NK-TS) were eluted from the gels and used in semi-quantitative absorption studies or were used to inhibit the formation of target–effector conjugates as an estimate of relative avidity. These findings were supported by a comparative analysis of cold target competition curves and saturation studies in which ^{51}Cr-labelled target cells were carefully titrated. The results suggest that NK cells 'mature' during ontogeny to higher avidity binding whereas the decline in NK function during senescence can solely be attributed to a decrease in population size. A comparison of high (CBA) and low (A/Sn) NK reactive strains revealed that in low responder (i) absolute NK frequency was decreased, (ii) relative NK-TS absorption per NK cells was low, and (iii) relative avidity of NK cells was identical to that in the high responder strain. These results suggest that the putative NK receptor to YAC may be of restricted heterogeneity.

Abbreviations: NK, natural killer cells; NK-TS, the target glycoproteins on the target cell which are recognized by NK cells; TBC, target-binding cell.

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INTRODUCTION

A recently described class of lymphocytes called natural killer (NK) cells has been shown to play an important role in tumour resistance *in vivo* (Kiessling & Wigzell, 1979). These cells arise in the bone marrow, develop independently of thymic influence and lack the characteristics of mature macrophages, B cells or T cells (reviewed in Kiessling & Wigzell, 1979; Herberman & Holden, 1977). NK cells are unique since they pre-exist at high levels in the host (Roder & Kiessling, 1978) in contrast to other effector mechanisms such as cytotoxic T lymphocytes (CTL) or antibody-dependent cell-mediated cytotoxicity (ADCC) which may require days or weeks to be primed. NK cells (Kiessling & Wigzell, 1979), therefore, might be expected to provide a first line of defence against newly arising malignancies. Hence it was important to establish the cellular basis for low NK reactivity in certain genotypes and in ageing mice in particular, since there is a concomitant increase in tumour susceptibility to NK sensitive tumours with age (Haller, Hansson, Kiessling & Wigzell, 1977). The spontaneous tumour incidence in some low NK reactive genotypes (SJL, AKR) is also

high whereas other low NK reactive strains (A/Sn, A/J) do not have enhanced tumour susceptibility possibly due to additional host resistance factors.

Some of the differences in NK function in low reactive genotypes and in old mice are clearly due to lower frequencies of NK cells (Roder & Kiessling, 1978). It is likely, however, that changes in binding characteristics may also play a role. It has previously been shown that certain glycoproteins isolated from the surface of target cells bind selectively to the surface of NK cells and specifically prevent subsequent recognition of the homologous intact target (Roder, Rosén, Fenyö & Troy, 1979a; Roder, Ahrlund-Richter & Jondal, 1979b). In the present study, this system is extended in an attempt to develop comparative absorption tests and inhibition assays of target–effector binding in mice of widely differing ages or genetic background.

MATERIALS AND METHODS

Mice

A/Sn, C57B1/6 and CBA mice were maintained in this laboratory by continuous single-line, brother–sister mating.

Tumour cell lines

Tumour cell targets were maintained by continuous *in vitro* culture. YAC is a T-cell lymphoma induced by Moloney leukaemia virus in A/Sn mice.

Nylon-wool columns

Monodispersed cells from various lymphoid organs were treated briefly (4 s) with H_2O 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.

Anti-T serum treatment

Nylon-wool column passed spleen cells were treated for 1 h at 4° with 1:15 rabbit anti-mouse thymocyte serum (Cedarlane Labs, Hornby, Ontario), washed and then treated with 1:10 guinea-pig complement for 45 min at 37°. This treatment killed 60–70% of the nylon-passed cells.

Target-binding cell assay

Lymphoid cells, depleted of erythrocytes by hypotonic shock were passed through nylon-wool columns and were labelled with fluorescein isothiocyanate (FITC, BDH Chemicals Ltd, Poole) as described previously, substituting BSS for saline (Roder & Kiessling, 1978). 2×10^6 target cells were then mixed with 2×10^5 FITC-labelled lymphoid cells in 0·2 ml culture medium and centrifuged at 200 *g* for 5 min, at room temperature in round-bottomed plastic tubes or microtitre plates. Tubes were placed on ice for 30 min and then aspirated five to ten times with a pasteur pipette. The percentage of fluorescing cells (effectors) binding to non-fluorescing cells (targets) was then determined after counting 300–400 effector cells under a UV microscope. Fortuitous associations were tested by tapping the coverslip. Variation between replicate samples was always less than 10%.

Cytolytic assay

2×10^4 target cells, labelled with [^{51}Cr]-sodium chromate as previously described (Kiessling, Klein & Wigzell, 1975), were incubated for 3 h at 37° with varying numbers of effector cells (normal unimmunized lymphocytes) 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 gamma counter. Spontaneous release was determined by culturing ^{51}Cr-labelled targets alone and total label was determined by counting an aliquot of target cells after resuspension in the microwells. The following formula was used to compute percentage lysis:

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

Isolation of NK-TS

The detailed procedures are described in Roder *et al.* (1979a, b). Briefly, YAC cells at a concentration of 2×10^8 ml were extracted with 4 mM Triton X−100 and the supernatant was reduced with mercaptoethanol and electrophoresed in 0·1% SDS and 8% polyacrylamide using the Laemmli technique. Following electrophoresis, gel slabs were washed for 24 h in buffer to remove most of the detergent but little if any of the proteins. Channels containing cell extracts or molecular weight standards were cut vertically and stained for protein with Coomassie brilliant blue. Additional channels containing cell extract were sliced horizontally in 3 mm sections. These slices were macerated in 0·1 ml buffer and incubated at least 24 h at 4°. Supernatants were pre-incubated with nylon-passed spleen cells and assayed for inhibition of TBC. Fractions inhibiting TBC were pooled from several gel

slabs and protein was measured by the Lowry technique.

RESULTS

Absorption studies

Triton-X-100 solubilized and reduced surface proteins from YAC cells were electrophoresed in SDS-polyacrylamide gels as previously described (Roder *et al.*, 1979a, b). The NK-target structures (NK-TS) were taken as those proteins eluted from gel slices which inhibited the binding of NK cells to intact YAC targets (Roder *et al.*, 1979a). The characteristic inhibitory bands, 140K, 160K and 240K ± 10K were tested separately or pooled as a source of NK-TS. Similar results were obtained with each preparation and therefore only the data with the 140K protein is reported. Spleen cells were passed over nylon-wool columns and treated with anti-T serum and complement in order to remove T cells. As shown in Fig. 1, spleen cells from high NK reactive CBA mice absorbed much more NK-TS than nylon-passed spleen cells from NK low reactive A/Sn mice ($P < 0.001$). Spleen cells pooled from CBA mice were much more effective at binding and lysing YAC targets than spleen cells from A/Sn mice (Fig. 2) as previously shown (Roder & Kiessling, 1978; Kiessling *et al.*, 1975). Similar results were obtained with non-T-cell depleted populations (data not shown). If the number of NK cells was estimated from the frequency of TBC (Roder & Kiessling, 1978; Roder, Kiessling, Biberfeld & Andersson, 1978) and the number of absorbing cells was equalized on this basis, then over twice as many NK cells from A/Sn compared to CBA mice were required to absorb 50% of the NK-TS activity from the SDS-PAGE eluted proteins (Fig. 1). In comparison, nylon-passed spleen cells from old (8 month) CBA mice had the same low frequency of TBC as A/Sn mice (Fig. 2) but absorbed the same amount of NK-TS as 6 week CBA mice on a per NK cell basis (Fig. 1). These results suggested that the low NK response in ageing CBA mice could be accounted for solely on the basis of a decreased popu-

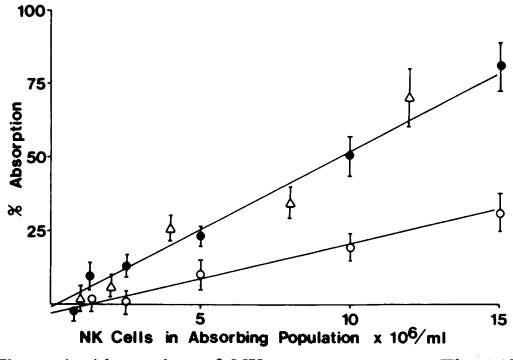

Figure 1. Absorption of NK-target structures. The 140K species of NK-TS protein from YAC cells was absorbed with nylon-column passed anti-T and complement-treated spleen cells from CBA or A/Sn mice of various ages. The number of NK cells in the absorbing population was calculated on the basis of the frequency of TBC which was 48%, 16% and 15·6% in 6 weeks CBA (●), 35 week CBA (△) and 6 week A/Sn (○), respectively. The absorbed NK-TS material was then tested for residual activity by inhibition of the TBC assay using YAC as target.

$$\% \text{ absorption} = 1 - \frac{\% \text{ inhibition of TBC with pre-absorbed NK-TS}}{\% \text{ inhibition of TBC with unabsorbed NK-TS}} \times 100$$

The frequency of TBC in nylon-passed anti-T and complement-treated 6 week old CBA spleen was 48·4% and 14·1% after a 1 h pre-incubation (4°) with buffer control or unabsorbed NK-TS, respectively. The values represent the mean ± SEM of three separate experiments. The correlation coefficient for both lines was $r > 0.9$ and the two slopes differed significantly in a Student's t test ($P < 0.001$).

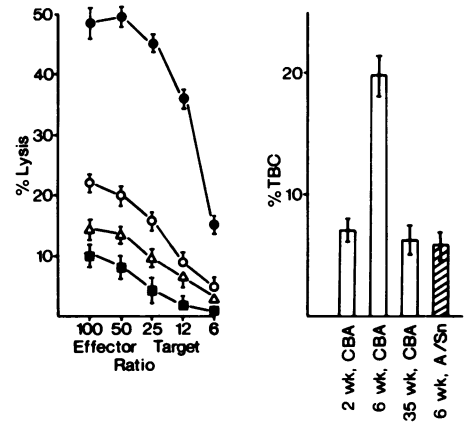

Figure 2. Target binding and lytic capacity of NK cells from mice of various ages and genotype. Spleen cells were pooled from eight mice in each group, passed over nylon-wool columns and tested in a 4 h cytolytic assay against YAC cells (left panel). Some nylon-passed spleen cells were fluorescein-labelled and mixed with a ten-fold excess of YAC cells to enumerate the percentage target binding cells (TBC, right panel). Values represent the mean ± SEM of triplicate samples. This experiment was repeated four times with similar results and the same relative differences were noted using nylon passed spleen cells treated with anti-T serum and complement. ○, 2 week CBA; ●, 6 week CBA; △ 35 week CBA; ■, 6 week A/Sn.

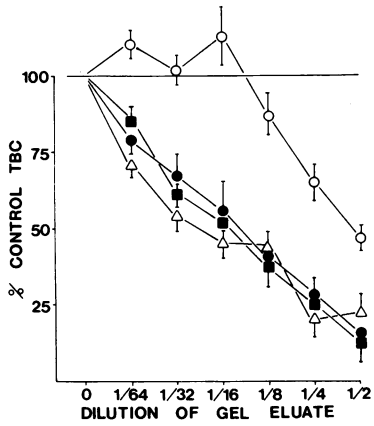

Figure 3. The relative avidity of NK cells. Spleen cells from mice of various ages or genotype were passed over nylon-wool columns and pre-incubated for 1 h at 4° with various dilutions of NK-TS isolated from YAC by SDS PAGE. The protein concentration in undiluted NK-TS preparations was 10 μg/ml. Spleen cells were then allowed to bind intact YAC cells and the frequency of TBC was enumerated. The values are expressed as % control TBC ± SEM of three separate experiments. Actual mean frequencies of TBC were 7·7%, 18·8%, 7·9% in 2(○), 6(●) and 35 (△) week old CBA mice and 8·0% in 6 week old A/Sn mice (■).

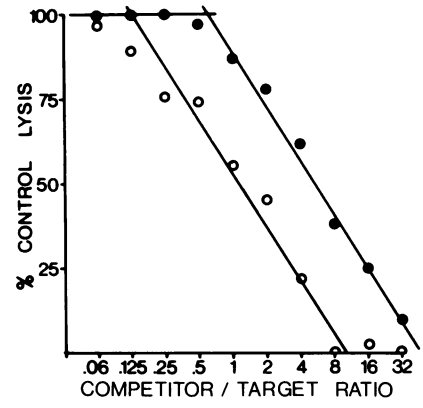

Figure 4. Cold target competition curves in different genotypes. Spleen cells were pooled from ten 6 week old mice in each group. Each microwell contained 10^6 spleen cells, 2×10^4 ^{51}Cr-labelled YAC cells and varying numbers of unlabelled YAC competitor cells in a 3 h cytolytic assay. Each point represents the mean lysis in quadruplicate wells. The standard deviation did not exceed 4% for any point. The data are plotted as % control lysis. Actual lytic values in control cultures with no competitor cells added were 63·7 ± 0·3% and 10·1 ± 0·3% for CBA (●) and A/Sn (○), respectively. The lines were plotted between the inflection points by the method of least squares. Values showing no inhibition or 100% inhibition were excluded from the analysis. This experiment was repeated five times with similar results.

lation size whereas additional factors such as the number of binding sites or binding avidity may be important in the low NK response in A/Sn mice.

NK avidity was estimated by determining the relative amount of NK-TS required for 50% inhibition of TBC. As shown in Fig. 3, a 1/16 dilution of NK-TS was required for 50% inhibition of TBC by both 6 week and 35 week old CBA mice whereas a 1/2 dilution was required to inhibit TBC to the same extent in 2 week old CBA mice. These results suggest that NK avidity in old, 35 week and adult, 6 week mice is the same, whereas the avidity in young, 2 week mice is substantially (eight-fold) less ($P < 0.001$). Inhibition curves from CBA and A/Sn mice overlapped, suggesting that NK activity was identical in high and low NK reactive genotype. Two different experimental systems were used to test this suggestion.

'Cold' target competition studies

Increasing numbers of unlabelled YAC cells were added to a constant number of ^{51}Cr-labelled YAC cells (2×10^4) and a fixed number of effector cells (10^6) in a competition type assay. As shown in Fig. 4, the first detectable inhibition of NK cytolysis occurred at a four-fold lower competitor/target ratio in A/Sn mice compared to CBA mice which would be expected if A/Sn effectors had fewer binding sites/cell as suggested in Fig. 1. The slopes of the competition curves, however, were parallel in CBA and A/Sn mice which suggests that the relative NK avidity may be similar in the two strains. If A/Sn had lower avidity receptors, then each increment of competitor cells would cause less inhibition in A/Sn than CBA and consequently the slope of the A/Sn curve would be expected to be less than the CBA curve, which is clearly not the case. Competition was not due to cell crowding in these experiments since the addition of equal numbers of glutaraldehyde fixed or unfixed thymocytes from 4 month old CBA mice caused no significant competition (data not shown).

A similar analysis was performed using effectors from very young (2 week), adult (6 week), or old (24 week) CBA mice. As shown in Fig. 5 (top panel) 6 week and 24 week old mice had a higher slope as expected from the experiments shown in Fig. 3, which suggested that NK cells from 2 week mice were of lower avidity.

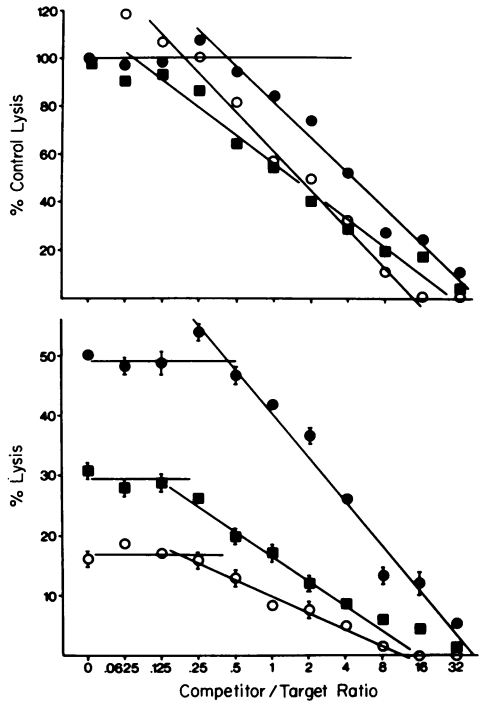

Figure 5. Cold target competition curves at different ages. Spleen cells were pooled from six CBA mice in the 2 week (■), 6 week (●) and 24 week (○) age groups. Competition was performed as described in Fig. 4. Data are plotted as actual mean % lysis ± SEM in triplicate wells (bottom panel) and as % control lysis (upper panel). Plotted lines were fitted by the method of least squares. In a regression analysis, $r = 0{\cdot}99$ for all three curves. This experiment was repeated three times with similar results.

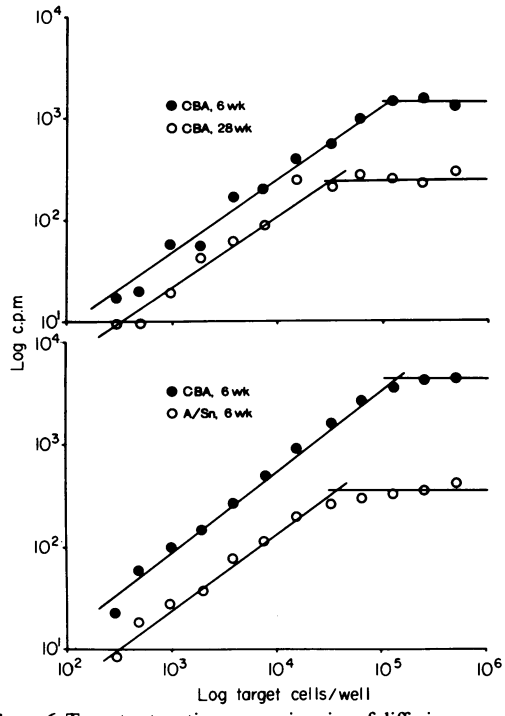

Figure 6. Target saturation curves in mice of differing age and genotype. Increasing numbers of ^{51}Cr-labelled YAC targets were added to 10^6 spleen cells/well and incubated for 4 h at 37° in a cytolytic assay. Spleen cells were pooled from six mice in each group. The values shown represent the mean c.p.m. released in quadruplicate wells co-cultured with spleen. SD did not exceed 5% for any point. The c.p.m. released by labelled YAC cultured alone have bee subtracted. This experiment was repeated four times with similar results.

Saturation studies

In these experiments, increasing numbers of ^{51}Cr-labelled target cells were added to a constant number of spleen cells in order to obtain saturation curves. As shown in Fig. 6, ^{51}Cr release from YAC cells progressed in a logarithmic fashion with increasing target cell concentration up to a characteristic inflection point after which no further increase occurred. This inflection point presumably represents the point at which all effector cells in the population are completely saturated by targets and since NK lysis follows one-hit kinetics this value will represent the number of effector cells/10^6 spleen cells, as shown previously in the cytolytic T lymphocyte system (Henney, 1971). These values are 14×10^4 and $3{\cdot}6 \times 10^4$ for CBA and A/Sn respectively, which is higher than the 2·4 and 0·8% estimates for NK cells made previously for CBA and A/Sn spleen cells on the basis of TBC frequency (Roder & Kiessling, 1978) and also somewhat higher than estimates made by applying Poisson statistics to a limiting dilution analysis (unpublished observation). However, the relative difference in NK frequency between CBA and A/Sn was the same in all three methods of analysis. The higher estimates for NK frequency using saturation studies probably reflect recycling of the effector to kill multiple targets during the incubation. The parallel slopes of the saturation curves (Fig. 6) indirectly supports the suggestion that NK avidity is similar in CBA and A/Sn mice. Similar results were obtained in 6 week and 28 week old CBA mice (Fig. 6, top panel).

DISCUSSION

These results suggest that the binding characteristics of the NK cell, as well as the frequency of NK cells, may be under developmental and genetic control in the mouse.

NK cells appeared at 1–2 weeks after birth, reached a peak at about 6 weeks of age and then declined gradually to low levels after 4 months (Fig. 2). This conclusion is based on (i) functional assay of cytolytic activity (Kiessling *et al.*, 1975), and (ii) measurement of the frequency of nylon-non-adherent lymphocytes binding to target cells (Roder & Kiessling, 1978). As discussed elsewhere (Roder *et al.*, 1978), it has been shown that the majority (> 80%) of the TBC measured represents functionally active NK cells and the putative recognition receptor can be specifically blocked by glycoproteins isolated from the surface of target cells (Roder *et al.*, 1979a, b). 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 crude radiolabelled target cell surface proteins are preferentially absorbed by NK cells, thereby eliminating the possibility that inhibition of the efffector resulted from a non-specific enzymatic-like activity in the various fractions (Roder *et al.*, 1979a). Furthermore, the absorption is selective since (i) surface proteins from NK insensitive targets are not absorbed by NK cells (Roder *et al.*, 1979a) and (ii) the quantity of antigen absorbed from sensitive targets varied directly with the concentration of NK cells in the absorbing population (Fig. 1). As yet, however, NK-TS has not been successfully isolated in sufficient quantity to allow high specific activity radiolabelling for detailed receptor studies. Therefore the NK-TS from YAC cells were used in an attempt to develop (i) comparative absorption tests and (ii) relative inhibition of TBC formation in order to give some indication of relative NK receptor density and avidity in different cell populations.

In the high reactive CBA strain we found that old (8 months) mice absorbed the same amount of NK-TS as 6 week old mice when the calculations were made on the basis of the NK cell frequency in the absorbing populations (Fig. 1). Similar results were obtained using 5×10^6 'immunoabsorbent' purified NK cells selected by binding to intact targets followed by velocity sedimentation and disruption of conjugates with EDTA as described in Roder *et al.* (1978) (data not shown). However, cell yields were too low using this method for extensive absorption studies. These results indirectly suggest that the density of NK binding sites may be similar in 6 week and 8 month old mice. In addition, the same quantity of NK-TS was required to inhibit 50% of the TBC in spleen cells from the 8 month and 6 week old groups (Fig. 3) which suggests that the relative NK avidity was also identical in the two groups. Therefore the low NK cytolytic response in old mice can solely be attributed to an age-dependent decrease in the size of the NK-cell pool as detected in the TBC assay (Fig. 2). It should be noted, however, that since interferon markedly stimulates NK cells in the mouse (Gidlund, Orn, Wigzell, Senik & Gresser, 1978), it is possible that low levels of endogenous interferon production in old mice, or decreased responsiveness to interferon, may account for part of the decline in NK activity.

The assumption that old mice did not have decreased NK avidity or number of binding sites was substantiated by two different approaches. In cytolytic assays, it was shown that the incremental addition of unlabelled target cells caused a parallel inhibition of observable lysis by spleen cells from 6 month and 6 week old mice (Fig. 5). Differences in NK avidity would have been expected to yield non-parallel inhibition curves which were not observed. Furthermore, when ^{51}Cr-labelled targets were carefully titrated it was observed that NK cells from 6 month and 6 week old mice exhibited parallel ^{51}Cr-release curves. If a threshold avidity is required to enable lysis of a target cell, then this result indirectly suggests a similar NK avidity in the two groups. Spleen cells from old mice were saturated by a lower number of target cells as would be expected since these populations contain fewer NK cells than found in 6 week mice.

The low cytolytic response of very young mice (2 weeks), unlike their old counterparts, was not only due to the low frequency of NK cells as measured in the TBC assay. The avidity of NK cells also appeared to be lower since (i) eight-fold more NK-TS was required for 50% inhibition of TBC and (ii) the slope of cold target competition curves was less in 2 week than 6 week old mice. NK-TS absorptions were not measured in these young mice. These results are compatible with the following hypothesis. During development NK cells rapidly expand from a pool of progenitors during the first few weeks after birth. During this time some maturation of the NK receptor occurs or alternatively there is a selection of higher avidity clones of NK cells. However, as yet, there is no convincing evidence that

NK cells are polyclonal in nature or that the recognition receptor is coded by V regions of immunoglobulin genes.

Surprisingly, a comparison of 6 week old mice from a low (A/Sn) and a high (CBA) NK reactive strain revealed that although the low reactive strain had fewer NK cells as expected, indirect estimates yielded similar NK avidities but different binding properties. Hence NK cells from A/Sn mice absorbed less NK-TS per effector (Fig. 1) whereas identical amounts of NK-TS were required for 50% inhibition of TBC (Fig. 3). Parallel cold target competition curves and parallel saturation curves (Fig. 6) further suggested, indirectly, that NK avidity in CBA and A/Sn mice was similar. These results indirectly suggest that NK receptors may be more restricted in heterogeneity than antibody-like receptors. Further analysis awaits the isolation of the NK receptor since masking of receptors cannot be ruled out as a contributing factor to apparent NK inefficiency.

In summary, these results suggest a maturation of putative NK receptors during ontogeny, whereas the decline in NK function in senescence can be attributed largely to a decreased effector population size rather than intrinsic cellular defects. Further support for this hypothesis is provided by the observation that NK cells from old mice or low reactive genotypes (A/Sn), are competent to be activated by interferon (Gidlund, personal communication). Comparison of high and low responder genotypes indirectly suggests that although NK binding properties differ, relative NK avidity is of much more restricted heterogeneity than would be expected if the antigen-combining sites on NK cells were coded by V regions of immunoglobulin genes.

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