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Missing self by heterogeneous natural killer cells

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Abstract. Some murine (YAC, P815 and SP20) and human (Molt4, Raji and HR7) tumour cell lines were (i) treated with IFN-γ for inducing enhanced expression of MHC class I antigen, or (ii) given a brief treatment with citrate buffer (pH 3.0), which resulted in denaturation of class I MHC antigens on these tumour cells. IFL-γ or acid treated tumour cells were used as unlabelled competing targets in cold target inhibition assays. The results indicated that the competing ability of acid-treated tumour cells remained unaltered, whereas IFN-γ treated tumour cells competed with significantly less efficiency. These results have been evaluated in light of the current view of NK cell development and the expression of inhibitory receptors for MHC class I molecules (IRMs), on NK cells. A modified view on NK cell heterogeneity based upon IRM expression has been proposed which reconciles several apparently discordant observations about the activity and role of NK cells. Two classes of NK cells have been proposed. Type I NK ceils have target recognition receptors which do not recognize autologous normal cells, lack IRMs, and may participate in first line of defence against transformed cells *in vivo.* Type II NK cells have target recognition receptors for autologous normal cells and express at least one self-reactive IRM in order to prevent auto-killing. Type II NK cells participate in killing those transformed cells which down-regulate their MHC class I expression in order to escape cytotoxic T-cell surveillance. It is also postulated that mechanism of inverse correlation of target cell MHC class I expression levels and their susceptibility to NK cells, involves interference model of missing self hypothesis for type I NK cells and inhibitory signal model of missing self hypothesis for type II NE cells. Finally, it is proposed that acid treatment of tumour cells enhances their lysis susceptibility by making them additionally susceptible to type II NK cells, rather than enhancing their killing by type I NK cells. This proposition would explain the lack of effect of acid treatment on the competing ability of tumour cells, when target cells are only lysed by type I NE cells.

Keywords. NK cells; MHC antigens; KIRs; interferon; immunesurveillance; LAK cells.

1. Introduction

Whereas cytotoxic T-cells recognition of targets cells requires the expression of autologous class I MHC molecules on the latter, there is no such MHC restriction in case of NK cell-mediated killing of target cells. Inverse correlation between the levels of MHC cells I antigens expressed on tumour target cells and their susceptibility to NK/LAK cells has however been reported for many tumour cell lines (Harel-Bellan *et al* 1986; De Fries and Golub 1988; Storkus *et al* 1989; Reiter and Rappaport 1993; Sarin *et al* 1993). Ljunggren and Karre (1990) had proposed two possible mechanisms for this phenomenon. Firstly, MHC I molecules on target cells may interfere with the recognition of the target by the effector NK cells (interference model), or MHC I molecules may send a negative signal to NK cells to down-regulate the lytic ability of these effector cells (inhibitory signal hypothesis). The inhibitory signal hypothesis

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received support from recent reports describing a variety of receptors for MHC class I molecules, on subsets of NK cells, which upon engagement with specific MHC I ligand on target cells, suppress the lytic activity of NK cells (Moretta *et al* 1993, 1996; Yokoyama and Seaman 1993; D'Andrea *et al* 1995). In the present paper, these receptors will be referred to as inhibitory receptors for MHC class I molecules or IRMs. Interference model has also found experimental support, from results of experiments in which effect of upregulation of MHC class I antigen expression on their ability to compete in cold target inhibition assays, has been studied (Haridas and Saxena 1995a, Saxena 1996). NK cell heterogeneity based upon IRM expression, has been described over last few years (Yokoyama 1995; Wagtmann *a al* 1995). Ly 49, a type II transmembrane proteins with extracellular C-type lectin domains, is expressed on a subpopulations of NK cells from C57 BI/6 mice. Engagement of Ly 49 molecule with H-$2^{k/d}$ class I MHC molecules on target cells, sends a turnoff signal to Ly 49^{+} NK cells, thus sparing the target from lysis by these effectors (Karlhofer *et al* 1992; Yokoyama and Seaman 1993). A similar heterogeneity in human NK cell population based upon the expression of a different type of receptor molecules (p58 family of molecules, belonging to immunoglobulin superfamily) for class I HLA molecules, has been demonstrated (Moretta *et al* 1993; Wagtmann *et al* 1995). Even though Ly 49 and p58 families of molecules are not homologous, their functions appear to be similar. Human homologue of Ly 49 and murine homologue of p58 molecules may eventually be found (Moretta *et al* 1994; Miyazaki *et al* 1996). It has been suggested that expression of IRMs would prevent the killing of normal autologous cells which express MHC I molecules. Only aberrant cells which switch off the expression of one or more types of MHC class I molecules, under the selection pressure of cytotoxic T-cell surveillance, will be susceptible to NK cell surveillance activity, because by switching off a particular class of MHC I molecules, these cells will be unable to send inhibitory signal to the NK cells which express IRM for that class of MHC I molecules. This reasoning has resulted in a view where normal NK cells must express at least one type of self-reactive IRM in order to prevent autoimmunity (Lanier and Philips 1996). This view on the role of IRMs is however not concurrent with the earlier view on NK cells having a role in immune surveillance against spontaneously transforming tumour cells or virus-infected cells (Herberman 1982; Saxena 1997), unless one assumes that freshly transformed cells must necessarily switch off the expression of at least one of their MHC class I molecules in order to become NK susceptible.

Suguwara *et al* (1987) have described a technique in which a brief exposure to pH 3·0 selectively abrogates class I MHC antigens from tumour cell surfaces. We have confirmed this finding and found an increased LAK susceptibility of pH 3·0 treated tumour cells (Darin and Saxena 1994; Haridas and Saxena 1995c; Saxena *et al* 1995). Moreover, interferon-induced decline in LAK susceptibility, which was accompanied with an increase in MHC class I antigen expression, was also restored to normal levels by pH 3·0 treatment (Sarin and Saxena 1994). In the present paper, we present data indicating that denaturation of class I MHC antigens on tumour target cells results in enhanced NK lysis but no effect on the ability of these tumour cells to compete in cold target competition assays. These results unlike our previous results on the effect of IFN induced increase in class I MHC expression, on their competition ability (Haridas and Saxena 1995a,b; Saxena 1996), can not be explained on the basis of interference model.Based upon our results and that of others, we have in the present communication, presented a modified view of ontogeny of NK cell IRMs, which reconciles many

apparently discordant observations. We have argued that both "inhibitory signal" and "interference" hypotheses may be valid explanations of inverse correlation, depending upon the type of effector NK cells. In addition, we propose that there may exist a NK cell population (type I NK cells) which expresses no IRM molecules, in addition to IRM expressing NK cells (type II NK cells), and that the two types of NK cells may have non-overlapping biological roles.

2. Materials and methods

2.1 *Effector and target cells*

Spleen cells from C57BL/6 mice and human peripheral blood lymphocytes from normal donors were used to generate murine and human IL-2 activated killer (LAK) cells respectively (Haridas and Saxena 1995a,b). In both the cases, cells were cultured at 5×10^6/ml with 200 U/ml of human recombinant IL-2 (HRIL-2) in complete medium. After two days, the cultures were split into two and supplemented with an equal volume of fresh medium and 100 U/ml of HRIL-2. Cells were harvested on day 5, washed and used as effectors in chromium release cytotoxicity assays. YAC (murine T-cell lymphoma), P815 (murine mastocytoma), SP20 (murine myeloma), MOLT-4 (human T-cell leukemia), HR7 [human gastric carcinoma and Raji (human B-cell leukemia)] cell lines were propagated in culture in RPMI-1640 supplemented with 5% FCS, 2×10^{-5} 2-mercaptoethanol, 300µg/ml of glutamine and 60µg/ml of gentamicin (complete medium). For acid (pH 3·0) treatment of the cell line, tumour cell pellets ($1–10 \times 10^6$) were suspended in 0·5 ml of cold 0·2 M citric acid–sodium phosphate, buffer of pH 3·0, containing 1% w/v of BSA. After a 5 min incubation at 4°C, cold complete medium was added in excess to neutralize the pH and cells were washed twice in fresh medium. For interferon treatment, tumour cells were cultured at $0·2 \times 10^6$ cells/ml with 200 U/m1 of human recombinant interferon- γ (IFN-γ, Biogene Research Corp., Cambridge, MA, USA) at 37°C for 48 h and washed.

2.2 *Estimation of relative levels of class I MHC antigens on tumour cells*

Expression of class I MHC antigens on tumour cells was assayed by a cell ELISA technique described by Sarin and Saxena (1990). Briefly, pelleted tumour cells were suspended in 0·1 ml of hybridoma supernatant (ATCC hybridoma HB 102 secreting antibodies to H-$2D^d$ molecule for murine cell lines, or ATCC hybridoma HB95 secreting a monomorphic monoclonal antibody to HLA-A,B and C molecules, for human cell lines) and incubated at 4°C for 30 min. At the end of the incubation, cells were washed thrice and suspended in 0·1 ml of 1:200 diluted rabbit antimouse IgG-HRPO conjugate and incubated at 4°C for 30 min. Orthophenylene diamine (OPD) was used as a substrate and colour development was monitored at 492 nm.

2.3 *Chromium release assay of cytotoxicity and cold target competition assays*

Procedure for chromium labelling of tumour cells and the chromium release assay of cytotoxicity has been described before (Saxena *et al* 1988). For competition assays, an

E/T of 55/1 was used because at this E/T, target lysis fell in linear range of the lysis curves for all targets examined. Along with the labelled targets, non-labelled tumour cells (control, pH 3·0 treated or IFN-γ treated competitors) were included into the cytotoxicity assay at competitor/target ratios of 8. Control lysis in the absence of competitor cells was also determined and the per cent inhibition of target lysis caused by the addition of competitor cells was calculated.

2.4 *Statistical analysis*

Paired *t*-test was employed to determine the level of significance of the differences in competing abilities of control, pH 3.0 treated and IFN-y treated tumour cells.

3. Results

3.1 *Modulation of class I MHC antigen expression on tumour cells*

The six tumour cell lines used in the present study, were subjected to (i) treatment with either murine (for murine cell lines) or human IFN-γ (for human cell lines), or (ii) pH 3.0 treatment, as described in §2, and tested for the expression of class I MHC antigens. Results summarized in table 1 indicate that IFN treatment resulted in a significant increase in the expression of MHC class I antigens (except on Raji cell line), whereas a significant loss of class I MHC antigen expression as a result of treatment with pH 3·0 buffer, was observed in all cases.

3.2 *Effect of modulation of expression of class I MHC antigens on the ability of tumour cells to compete in cold target competition assays of cytotoxicity*

Competitive inhibition of lysis of P815 cells by control, IFN-γ or acid-pH-treated, unlabelled P815, SP20 and YAC cells, was studied at a competitor/target ratio of 8. Similarly, competitive inhibition of lysis of Raji cells by control, IFN-γ or acid-pH-

Table 1. Effect of treatment with IFN-y or citrate buffer pH 3·0, on the expression of MHC class I antigens on some tumour cell lines.

	MHC class I expression (ELISE absorbance/10^6 cells)		
Cell line	Control cells	pH 3·0 treated cells	IFN-γ treated cells
YAC	0·38 ± 0·02	0·10 ± 0·01	1·13 ± 0·10
P815	0·97 ± 0·10	0·37 ± 0·06	1·32 ± 0·10
SP20	0·70 ± 0·05	0·29 ± 0·06	1·25 ± 0·10
Molt4	0·63 ± 0·02	0·15 ± 0·01	1·12 ± 0·08
Raji	0·91 ± 0·06	0·23 ± 0·03	0·97 ± 0·07
HR7	0·97 ± 0·04	0·23 ± 0·02	1·77 ± 0·05

Different tumour cell lines were subjected to treatment with IFN-y or to a brief treatment with citrate buffer pH 3·0 and expression levels of MHC class I molecules on control and treated cells were determined as described in § 2. Each value is a mean + SEM of 6 to 10 determinations.

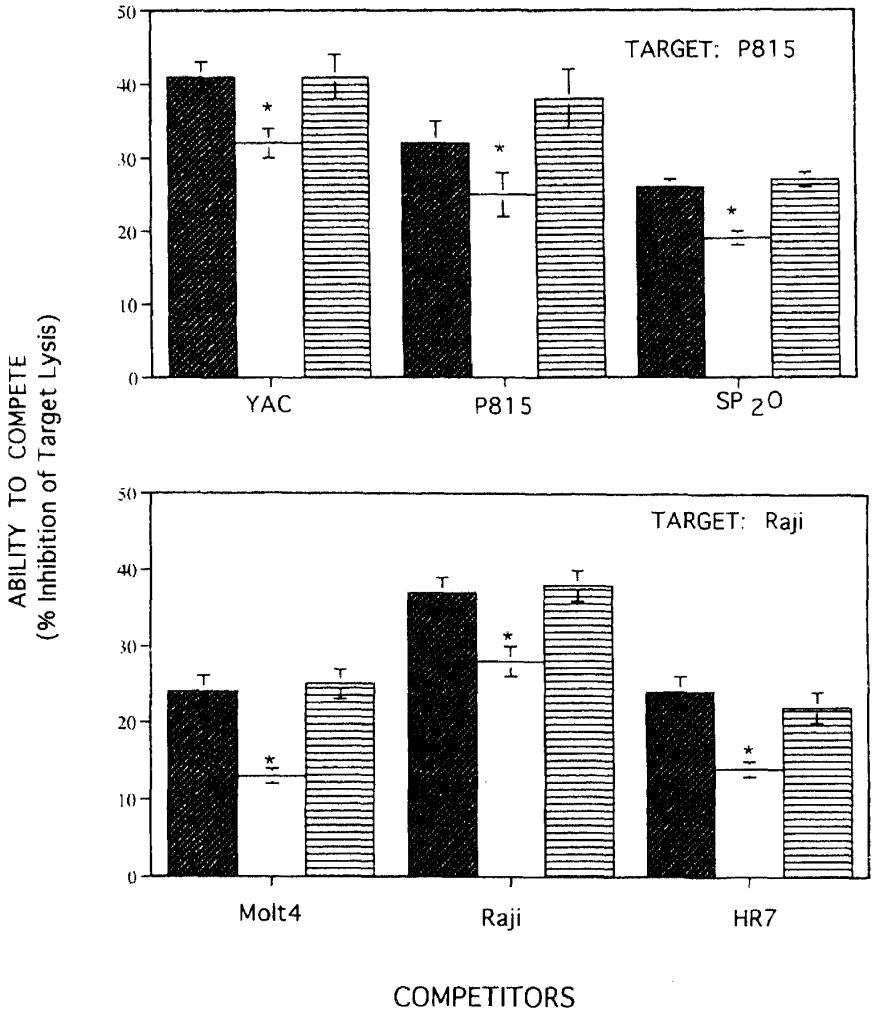

Figure 1. Cytotoxicity assays were performed using P815 (top panel) or Raji (bottom panel) target cells and mouse or human 1L2 activated NK effector cells respectively. Different competitor cell lines (control, IFN-γ-treated pH 3·0 treated) were added at competitor/target ratios of 8 and inhibition of target lysis was computed for all control or treated competitors, as described before (Haridas and Saxena, 1995a,b). In all cases, IFN-γ-treated competitors induced significantly lower competitive inhibition of the target lysis (*P < 0·05), where as pH 3·0 treated competitors competed as well as the untreated competitors. Error bars denote SEM of results obtained in 5 to 7 experiments in each case. For all tumour cells used in these experiment, IFN treatment induced significant NK resistance and pH 3.0 treatment enhanced the NK susceptibility (Haridas and Saxena 1995 a,b,c; Saxena *et al* 1995). (□), Control competitor; (□), IFN-treated competitor; (□), pH3 treated competitor.

treated, unlabelled Raji, Molt4 and HR7 cells, was also studied. A summary of all these experiments is shown in figure 1. These results indicate that for each target-competitor combination, competitor cells pre-treated with acid pH, induced competitive inhibition comparable to control untreated cells. Competitor cells pre-treated with IFN-γ however induced significantly lower inhibition of target lyses and this change in competing ability was statistically significant in all cases.

Discussion

Demonstration of inhibitory receptors of MHC class I molecules (IRMs) on NK cells over last several years has created a great deal of excitement in the area of NK cell biology (Moretta *et al* 1993; Yokoyama and Seaman 1993; D'Andrea *et al* 1995). Even before the actual demonstration of IRMs on NK cells, presence of such receptors had been postulated, in an effort to explain the inverse correlation between the levels of MHC class I antigen expression and the NK susceptibility of target cells (Ljunggren and Karre 1990; Liao *et al* 1991). The biological rational for this inverse correlation, and therefore of the presence of IRMs on NK cells, was as follows. Tumour cells *in vivo* are subject to cytotoxic T-cell-mediated immune surveillance and in order to escape this surveillance, tumour cell variants may arise which down-regulate their expression of MHC class I molecules. Immune system deals with such variant tumour cells by having in its repertoire NK cells, which can not kill normal autologous cells expressing full library of MHC class I molecules, but can kill tumour cell variants lacking one or more MHC class I molecules. IRMs on NK cells serve the function of sensing MHC class I molecules on target cells. There appears to be a marked heterogeneity within NK cell population based upon the expression of IRMs (Yokoyama and Seaman 1993; Moretta *et al* 1996). All NK cells do not express all IRMs but all NK cells must express at least one of the IRMs, recognizing one of the self MHC class I molecules, to prevent the killing of normal autologous cells by NK cells (Lanier and Philips 1996). The view is further extended to postulate that during the ontogeny of NK cells, only those NK cells are allowed to develop which express at least one self-reactive IRM (Lanier and Philips 1996). This view however poses several problems. (i) NK cells are known to be able to kill syngeneic tumour cells. This can not be explained until unless one makes an assumption that all syngeneic tumour cells must be deficient in expression of at least one of the MHC class I molecules. There is no evidence for such a generalization. (ii) By the same argument, NK cells can not have a role in killing freshly arising tumour cells *in vivo* or virus infected target cells (a first line of defence role, that has historically been ascribed to NK cells, Herberman 1982), unless a simultaneous loss of expression of at least one MHC class I is postulated on such cells. (iii) It is believed that a target cell expressing a particular MHC class I molecule may not be killed by NK cells expressing IRM reactive to that MHC molecule, since presence of such interactive receptor ligand interaction would "globally" turn off the NK cell (Yokoyama and Seaman 1993; Yokoyama 1995). If this assumption is correct, it becomes difficult to explain how does a NK-susceptible target cell acquires NK-resistance as a result of upregulation of MHC class I expression. This is because NK susceptibility indicates no interaction of NK cell IRMs with target MHC class I molecules in the first place, and increased MHC class I expression should not qualitatively after the MHC-IRM interaction from a non-interactive one to an interactive one. (iv) Finally, our results indicating that MHC class I upregulated tumour cells compete poorly in cold target competition assays, are better explained by considering "interference hypothesis" rather than "inhibitory signal hypothesis" as a model to account for inverse correlation between NK susceptibility and expression levels of MHC class I molecules on target cells (Haridas and Saxeria 1995a,b; Saxena 1996).

I would like to introduce a modification of the current view of NK cells and IRM expression, which appears to explain the above mentioned discrepancies, and also explains the results in this paper which show that acid treated tumour cells have

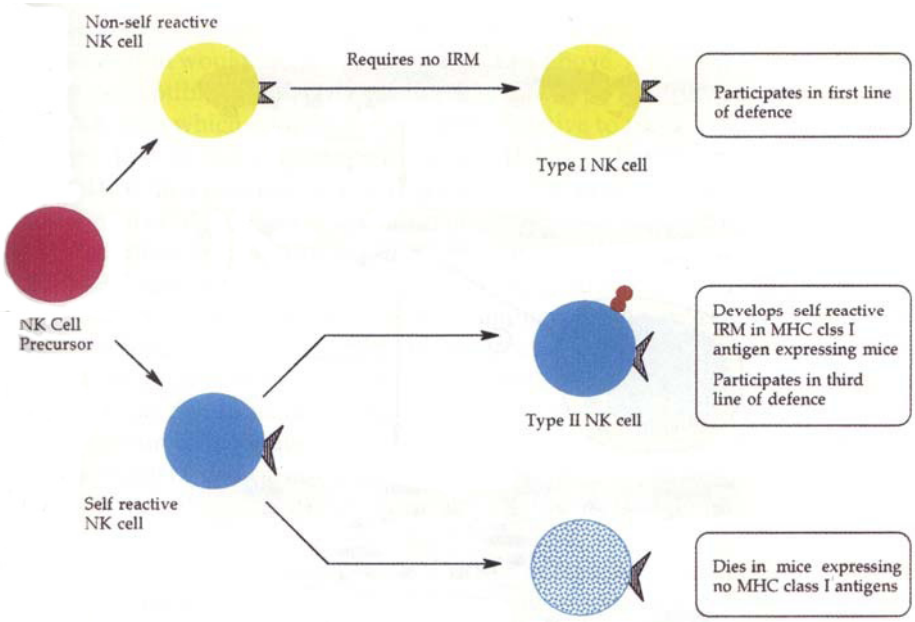

Figure 2. A modified view of ontogeny of IRMs on LK cells. During development, if a NK cell expresses a target recognition receptor which recognizes nothing on autologous normal cells, the cell develops without expressing any I R M (type I LK cell), and participates in the first line of defence. If NK cell happens to have a receptor recognizing some ligand on normal autologous cells, this cell expresses one or more IRMs (type II NK cell) in normal mice expressing class I MHC antigens, and participates in the third line of defence. In mice expressing no class I MHC antigens (/3, microglobulin gene disrupted mice), type II NK cells may not develop.

enhanced NK susceptibility but their ability to compete in cold target inhibition assays, is not changed. In the modified view, expression of IRMs on NK cells during their ontogeny, depends upon the kind of target recognition receptors which they possess (figure 5). If a given NK cell has a target receptor which does not recognize any molecule on autologous normal cells, such NK cell poses no potential danger of auto-killing, and expresses no IRMs, as it needs none. Let us term these NK cells as *type I NK cells.* On the other hand, NK cells which recognize target structures on autologous normal cells, are induced to express at least one self reactive IRM in order to prevent auto-lysis. Such NK cells may be called *type II NK cells.* Type I NK cells would be able to kill spontaneously arising tumour cells as well as virus-infected autologous cells which express new target structures recognized by type I NK cells, even if these target cells have not switched off or down-regulated their expression of class I MHC molecules. Killing of syngeneic cell lines by NK cells may thus be ascribed to type I NK cells. Type I NK cells may also participate in first line of defence against transformed autologous cells. After transformation however, the cells come under the immune-surveillance of cytotoxic T-cells (second line of defence), and in due course of time some of these cells may down-regulate the expression of some of their class I MHC molecules, in order to escape the T-cell surveillance pressure. MHC I down regulation now renders such cells susceptible to the third line of defence by type II NK cells, since inhibition inducing MHC class I molecules are no longer expressed on these trans-formed cells. This model explains the lysis of syngeneic tumour cells as well as

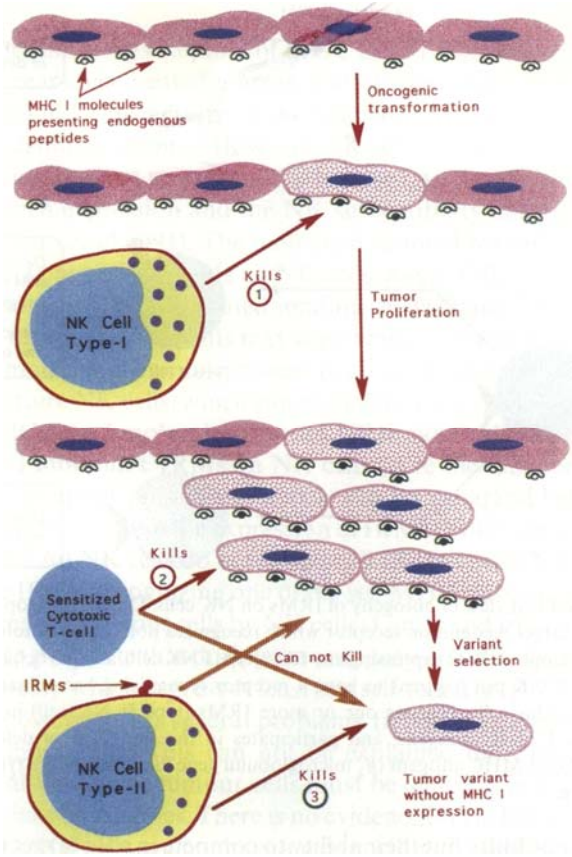

Figure 3. Proposed scheme for the involvement of type I and type II NK cells in immune-surveillance against transformed cells. When a cell transforms, it may, like a normal cell, express the complete library of MHC class I molecules along with some novel transformation related antigens (displayed as filled circles in crescentic MHC class I molecules). Such a cell can not be lysed by NK cells bearing self-reactive IRMs. As a first line of defence, type I NK cells can lyse such freshly transformed cells. In second line of defence, cytotoxic T-cells would mediate a cytotoxic response against the transformed cells. In order to survive the onslaught of cytotoxic T-cells, some transformed cells may generate variants which switch off or down- down-regulate regulate the expression of one or more MHC class I molecules. Such variant cells may be killed by type II NK cells in a third line of defence. Numbers in circles refer to lines of defence.

transformed autologous cells, by type I NK cells. It is possible that the development of IRM bearing type II NK cells does not take place in animals which express no MHC class I antigens (e.g.,$ß_2$ microglobulin gene disrupted mice), as that would explain why cells from $ß_2$ microglobulin deficient mice kill tumour targets like YAC (due to the presence of type I NK cells), but do not lyse normal Con-A blasts from $ß_2$ micro-globulin-deficient mice (Hoglund *et al* 1991). A second postulation I would like to make is that type I NK cells, while having no IRMs, may none-the-less kill MHC class I up-regulated tumour cells with lower efficiency, due to interference caused by MHC

class I molecules, in target recognition (i.e., the interference model refered to above). This postulation would nicely explain the point 3 above. A tumour target cell which is basically susceptible to NK cells must be killed by type I NK cells as well as by those type II NK cells which do not have any IRM reactive to the tumour cell MHC class I molecules. For all practical purposes, a type II NK cell expressing a irrelevant (non reactive) IRM for a given target cell, is like a type I NK cell for this particular target cell. Upregulation of MHC class I expression makes it less susceptible to lysis by type I NK cells, by interference mechanism. On the other hand, acid pH treatment denatures the class I MHC molecules on tumour cells, making it additionally susceptible to type II NK cells which failed to lyse the untreated tumour cells due to MHC-IRM interaction. Since enhanced susceptibility of acid-pH-treated tumour cells is not due to an increased interaction of treated tumour cells with the effectors which were originally killing it, but is due to its additional killing by type II NK cell population, the acid-treated tumour cells would not compete more efficiently in cold target inhibition assays, in which target lysis is by type I NK cells. This explains our current observation of a lack of modulation of competing ability of acid-treated tumour cells.

Note added in proofs: Inhibitory receptors for MHC class I molecules on NK cells, referred to as IRMs in this paper, have recently been re-named as killer-cell inhibitory receptors or KIRs (*Immunology Today,* 17, 100, 996)

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