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Natural killer cells in peripheral blood and the mixed lymphocyte response: interaction with the transferrin receptor

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SUMMARY

Several reports suggest that natural killer (NK) cells recognize the transferrin receptor (TFR) as a target for killing, and that natural cytotoxicity may be involved in the control of stem cell proliferation in bone-marrow. This study tested whether NK-cell recognition of the TFR on activated lymphocytes plays a role in the control of peripheral immune responses. Six lymphoid lines were created from a single individual, and used as targets for cytotoxicity assays, using either peripheral blood mononuclear cells, or mixed lymphocyte reaction (MLR)-derived effectors. The cells responsible for killing were predominantly Leu-11$^+$Leu-7$^+$ NK cells, though CD3$^+$ cells accounted for about 25% of cytotoxicity from MLR. No correlation was observed between TFR density and NK susceptibility when using all six cell lines. Specifically increasing the density of TFR on a single cell line failed to increase susceptibility to NK, suggesting that the TFR does not act as a major target for natural cytotoxicity directed at lymphoid cells. Furthermore, the relatively low levels of killing observed indicate that activated NK populations that accumulate at sites of immune response are unlikely to play a direct immunoregulatory role.

INTRODUCTION

Natural killer (NK) cells are a lymphoid population which is principally identified by its ability to kill allogeneic tumour-cell lines without prior sensitization. The physiological role of NK cells is unclear at present. Classically they have been ascribed a role in tumour surveillance (Roder & Pross, 1982) but often they are unable to lyse freshly isolated autologous tumours (Vankey *et al.*, 1980). Recent work suggests that NK cells may play a part in the control of stem-cell proliferation in bone-marrow (Holmberg, Miller & Adult, 1984; Mangon *et al.*, 1984). Haemopoietic stem cells, and indeed neoplastic cells, express transferrin receptors (TFR), the principal means of incorporating iron into cells (Barnes & Sato, 1975; Phillips & Azari, 1975). Iron is essential for cell function, being a component of many enzyme systems, and may also play a role in regulating DNA synthesis via the rate-controlling enzyme ribonucleotide reductase (Reichard & Ehrenberg, 1979; Thelander, Graslund & Thelander, 1983).

Several reports have suggested that NK cells may recognize the transferrin receptor as a target for killing (Vodinelich *et al.*, 1983; Newman, Warner & Dennert, 1984; Lazarus & Baines, 1985). The present study was undertaken to determine whether NK-cell recognition of the TFR on activated lymphocytes plays a role in controlling the peripheral immune response. Tartof *et al.* (1984) identified activated NK cells capable of lysing Daudi cell targets in a peripheral immune response to Purified Protein Derivative. NK-cell activation is known to involve the T-cell derived lymphokines IL-2 and IFN-γ. We have previously demonstrated IL-2-dependant activation of TFR$^+$ NK cells expressing the phenotype Leu-11$^+$ Leu-7$^+$ M1$^+$ CD2$^+$ CD3$^-$ CD4/8$^-$ within allogeneic mixed lymphocytes cultures (Salmon *et al.*, 1985; Salmon & Bacon, 1986). Natural cytotoxicity has previously been observed functionally in MLR (Lopez-Botet *et al.*, 1982), though this phenomenon was believed to be T-cell mediated (Masucci, Klein & Argors, 1980) and the cells involved termed activated lymphocyte killers (ALK). However, recent studies by Phillips & Lanier (1986) have suggested that the ALK phenomenon observed in MLR is principally an effect of activated NK cells, rather than of a non-specific cytolytic T-cell population. In this study we stimulated peripheral blood mononuclear cells in one-way allogeneic MLR, and used the activated populations as effector cells to investigate the cytotoxic recognition of transferrin receptors on autologous lymphoblastoid cell lines, in order to determine whether such a process may play an important role in immunoregulation.

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MATERIALS AND METHODS

Generation of target cells—EBV-transformed lymphoblastoid cell lines

Infective Epstein–Barr virus was obtained from the adherent myeloid line B958. Peripheral blood mononuclear cells (PBMC), 10^7, from a normal healthy individual were resus-

pended in 250 μl diluted virus (determined by preliminary trials), and incubated for 1 hr at 37° in a gassed (5% CO_2) humidified incubator. The cells were then diluted to a concentration of 2×10^6/ml in RPMI containing 10% heat-inactivated fetal calf serum, glutamine and antibiotics, and also 1 mg/ml cyclosporin A. Aliquots, 200 μl, of these cell suspensions were placed in 96-well flat-bottomed microtitre plates (Flow Laboratories, Irvine, Ayrshire), and incubated at 37° for 7 days. The wells were subcultured, and six lines were grown from the transformed cells of a single individual (Bird, McLachlan & Britton, 1981). All of the lines proved negative for mycolasma contamination.

Generation of cytotoxic effectors in mixed lymphocyte culture

PBMC from two normal individuals were separated and resuspended to a concentration of 3×10^6/ml in RPMI containing 10% heat-inactivated fetal calf serum, glutamine and antibiotics. The stimulator population was exposed to 3000 rads gamma irradiation from a ^{60}Co source, then washed and resuspended in fresh medium. The non-irradiated responder population was, in all cases, derived from the same individual used to create the lymphoblastoid cell lines.

Both populations of cells were mixed together in equal proportions and placed into a number of 30 ml tissue culture flasks (15 ml per flask). The basal area of the flasks was 50 mm^2, thus ensuring intimate cell contact. MLR were cultured for 6 days prior to testing in cytotoxicity assays. Effector to target ratios (E:T) were determined from the number of viable cells in culture, using trypan blue exclusion.

Cytotoxicity assays—preparation and labelling of target cells

Effector populations were either fresh PBMC or MLR-derived effectors from the same individual used to generate the six EBV transformed target lines. Target cells were the six EBV lines: IAN-1–7 (excluding 4). Preliminary trials using these lines and K562 cells suggested that the targets must be in log-phase growth to obtain reproducible cytotoxicity. For this reason the IAN cell lines were subcultured 72 hr before each assay. Targets were labelled with ^{111}In oxine (Frost, Smith & Frost, 1978). Briefly, 10^6 cells were removed from the culture flasks, washed twice in PBS (pH 7·4) and resuspended in 200 μl PBS. ^{111}In oxine (Amersham Int. Aylesbury, Bucks) 5 μCi, were added to the target cells, which were then flicked gently to disperse the label. After 4 min incubation at room temperature, the cells were diluted to 10 ml with Hanks' BSS, pH 7·4, containing $NaHCO_3$ and 10% fetal calf serum. One-hundred microlitres of the cell suspension were removed (sample A), the target cells centrifuged for 5 min at 600 *g*, and an aliquot of the supernatant removed (sample B). Labelling efficiency was calculated in all cases by counting the two samples in a 1260 multigamma II counter (LKB, Croydon, Surrey), programmed for ^{111}In, for 30 seconds per sample and applying the following formula:

$$\text{Labelling efficiency} = 100 - \frac{\text{B(c.p.m.)}}{\text{A(c.p.m.)}} \times 100.$$

Target cells with labelling efficiencies of less than 50% were not used for cytotoxicity assays.

Cytotoxicity titration

All assays were performed in 96-well V-bottomed plates (Flow Laboratories). The medium used throughout was Hanks' BSS, pH 7·4, containing $NaHCO_3$ and 10% fetal calf serum. Effector cells were titrated across the plate in triplicate, leaving 100 μl of cell suspension in each well. Four dilutions were used in each assay, such that the addition of 10^5 target cells in 100 μl of medium resulted in effector to target ratios of 100:1, 50:1, 25:1 and 12·5:1. Minimum-release wells contained 10^5 target cells in 200 μl of medium, whilst maximum release was determined from similar wells containing 0·5% Triton X-100 (final concentration). Plates were incubated at 37° for 4 hr exactly. Following this incubation, they were centrifuged for 5 min at 600 *g*, then 100 μl aliquots of each supernatant placed in plastic LP3 tubes for gamma counting (30 seconds in a multigamma counter programmed for ^{111}In). Percentage release (cytotoxicity) was calculated as:

$$\% \text{ release} = \frac{E - C}{M - C}$$

where E = mean of experimental triplicates; C = mean of minimum release triplicates; and M = mean of maximum release triplicates.

Depletion experiments

Effector populations used in these studies were either fresh PBMC or MLR cells cultured at Day 6. Effector cells, $1{\cdot}5 \times 10^6$, were aliquoted into each of six LP3 tubes, and resuspended to 200 μl in Hanks' BSS containing 2% BSA. The effector aliquots were then depleted of various subpopulations by incubation with monoclonal antibodies and complement (Oda & Puck, 1961). The monoclonal antibodies used were OKT3, OKT4, OKT8 (Ortho Diagnostics, Raritan, NJ), Leu-11 and Leu-7 (Becton–Dickinson, Mountain View, CA). The remaining tube received PBS and complement as a control.

The resulting depleted effector cells were then tested in the cytotoxicity assay at E:T ratios of 100:1 and 50:1, based on the number of cells in the control aliquot. The contribution to the total cytotoxicity of cells expressing each of the tested antigens was calculated by subtracting the percentage release of the depleted population from that of the undepleted control.

Transferrin-binding assays

Human transferrin was prepared using the method of Morgan (1964). Radiolabelling of the protein was achieved using the method of David & Reisfeld (1974). Two forms of transferrin-binding analysis were performed: a titration pattern using Scatchard analysis (Scatchard, 1959) to determine receptor affinity; and a single point saturation binding analysis to determine receptor numbers. For the titration, 250 μl of cell suspension containing between 10^5 and 5×10^5 cells, were placed in LP3 tubes at 0° (on ice). ^{125}I-transferrin was added to the cells to give a final concentration in 0·5 ml of 2, 5, 10, 20, 50, and 100 nM. Duplicate assays were performed, with a third tube containing a 50-fold excess of unlabelled transferrin, to control for non-specific binding. The tubes were incubated on ice for 90 min, then washed three times in ice cold PBS by centrifugation at 600 *g*. The cells were transferred to fresh LP3 tubes and counted in a multigamma counter programmed for ^{125}I (60 seconds per sample). Specific activity of ^{125}I-transferrin was determined on each occasion. Non-specific binding was subtracted from the mean value of each duplicate pair, and the result expressed in nM/l transferrin bound. Scatchard plots were prepared, and the

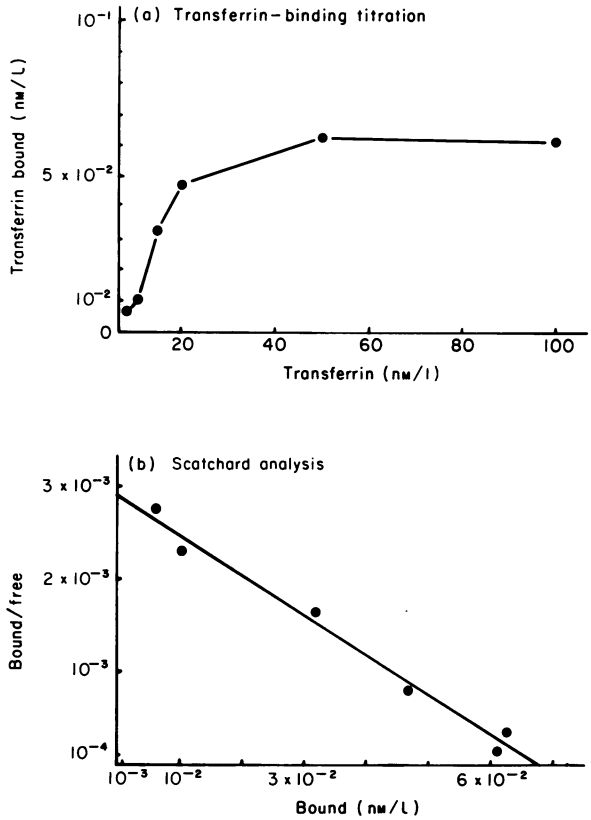

Figure 1. A representative transferrin binding analysis using the EBV-transformed lymphoblastoid cell line IAN-6. (a) A transferrin binding curve, with clearly saturable binding; (b) a Scatchard plot of the same data, relating bound transferrin to the ratio of bound/free. The slope of the line indicates the association constant (Ka) of the receptor.

receptor affinity (Ka) was determined from the slope of the regression line:

$$\text{Ka } (\text{lM}^{-1}) = \frac{y \text{ intercept}}{x \text{ intercept } (\text{Ml}^{-1})}.$$

Saturation-binding analysis

Provided only a single receptor of uniform affinity is present, a simple method of analysis may be used to determine the number of transferrin receptors present on cells. The single point analysis used here was performed in exactly the same way as the titration described previously, but only one concentration of transferrin was used (100 nM). Cell concentrations were between 0·2 and 1×10^6/ml. Corrected values of bound transferrin (ng/ml) were divided by the molecular weight of transferrin (80,000) and multiplied by Avogadros' number, to define the number of molecules bound per millilitre. The resulting figure was then divided by the cell count to give the mean number of receptors per cell.

Transferrin receptor modulation

The target cell line IAN-6 was cultured in four separate flasks for 2 days in RPMI containing 10% heat-inactivated fetal calf serum in a volume of 40 ml, with an initial seeding of 10^5 cells/ml. Twenty millilitres of medium were removed from each flask, and replaced with the same volume of fresh medium. One flask was used as a control, the other three received desferrioxamine (DFX) (Ciba–Geigy, Basel, Switzerland) in various doses to give a final concentration of 2, 5 and 10 μM. The cells were cultured for a further 48 hr before testing (Mattia *et al.*, 1984).

Table 1. The association constants (Ka) of transferrin receptors expressed by six EBV-transformed lymphoblastoid cell lines, measured by Scatchard analysis of transferrin-binding data in two separate experiments

Cell line	Ka (exp. 1) $\times 10^8$ l M^{-1}	Ka (exp. 2) $\times 10^8$ l M^{-1}
IAN-1	0·36	0·43
IAN-2	0·32	ND
IAN-3	0·52	0·36
IAN-5	ND	0·50
IAN-6	0·52	0·34
IAN-7	0·29	0·46

ND, Not done.

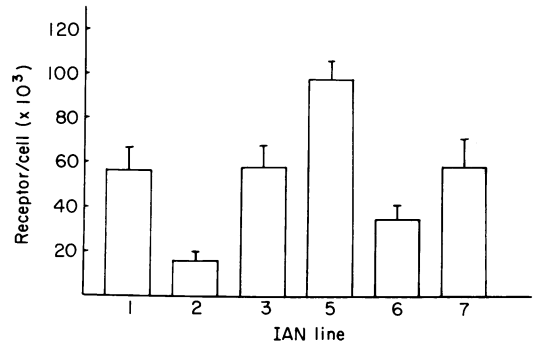

Figure 2. The mean number of transferrin receptors expressed during log-phase growth by six EBV-transformed lymphoblastoid lines (IAN). Mean and SD of six experiments.

RESULTS

Generation of six EBV-transformed lymphoblastoid cell lines (IAN) and assessment of their transferrin receptors.

Six EBV-transformed lymphoblastoid cell lines were generated from a single individual. These lines (IAN1-7, excluding 4) were assessed for TFR expression and affinity by transferrin binding titration. Figure 1 shows a detailed analysis of one of these lines. Transferrin binding was saturable, and Scatchard analysis showed a single straight line, indicating only a single species of receptor. All of the IAN lines similarly showed only a single straight line. Table 1 shows the affinity of the receptor assessed by this method on all six lines over two experiments. The affinities are all quite similar, within the expected variation for experimental error. This data suggests strongly that there is a single uniform species of receptor on all of the lines, and therefore indicates that a single point saturation analysis should be an accurate means of determining receptor number.

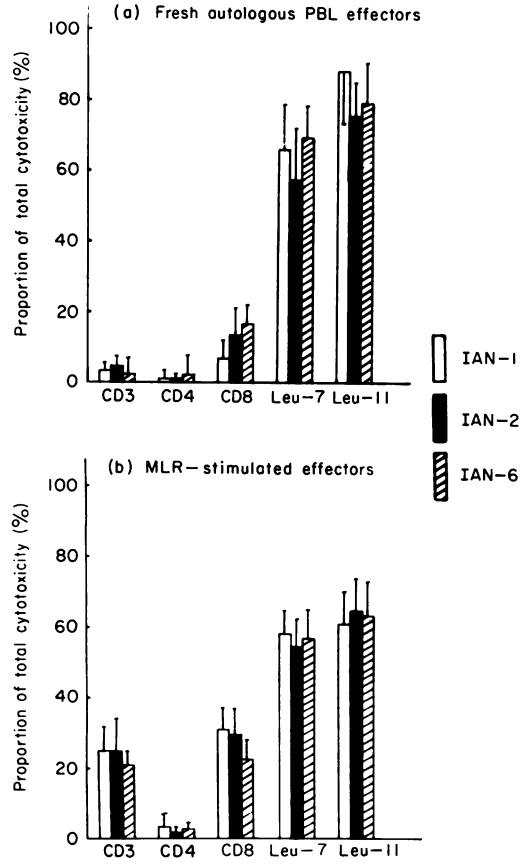

Figure 3. Characterization of cytotoxicity against three of the EBV-transformed lines (IAN-1, 2 and 6), using fresh PBMC effectors (upper) and MLR-derived effectors (lower graph). Mean results from E:T ratios of 100:1 and 50:1 respectively. Values represent the mean and SD of three experiments.

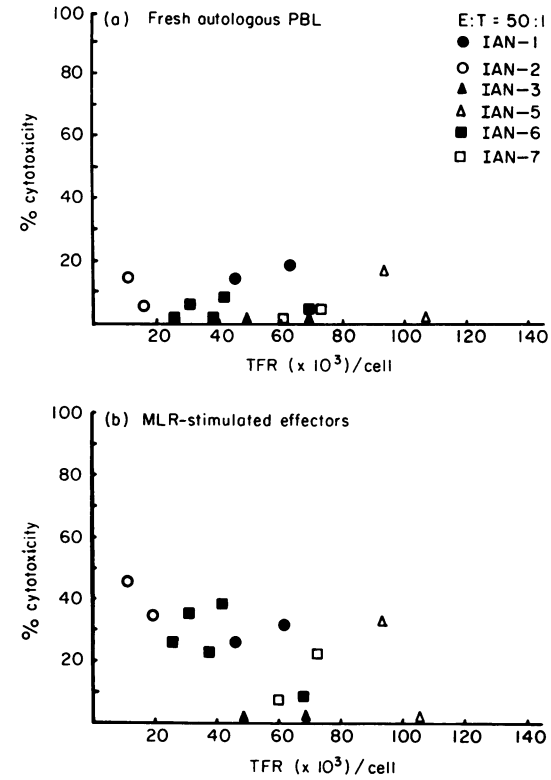

Figure 4. The relationship between transferrin receptor density and NK susceptibility of six EBV-transformed lymphoblastoid cell lines. The graph represents cytotoxicity using (a) fresh PBMC effectors ($r = -0{\cdot}029$), (b) MLR-derived effector cells ($r = 0{\cdot}48$); t-test analysis of the product moment correlation coefficients showed that neither of the correlations was significant.

The IAN lines showed a wide variation in mean transferrin receptor density, measured during log-phase growth, from 17,486 ± 4268–98,561 ± 9642 receptors per cell (Fig. 2). They were, therefore, highly suitable for the purpose of relating TFR density to NK susceptibility.

Characterization of cytotoxic effectors against IAN cell lines

Depletion experiments were performed to determine the contribution of cells expressing particular surface antigens to the killing of IAN cell lines. Autologous PBMC and MLR-derived effectors were characterized against three of the IAN lines: IAN-1, IAN-2, and IAN-6, at E:T ratios of 100:1 and 50:1 (Fig. 3). Killing by PBMC was almost entirely mediated by cells expressing the NK antigens Leu-11 and Leu-7. Most cytotoxicity by MLR-derived effectors was again mediated by Leu-11- and Leu-7-bearing cells, though about 25% of killing was performed by $CD3^+$ $CD8^+$ cells. These results indicate quite clearly that the killing in both systems is mediated predominantly by NK cells. Similar experiments were performed using K562 and Daudi cell targets. Using this depletion technique the effector populations showed the same antigen distribution as for IAN cell targets, both with fresh PBMC and also MLR-derived effectors (data not shown).

Relationship of surface transferrin receptor density to NK susceptibility of six EBV-transformed lymphoid cell lines (IAN)

Cytotoxicity assays were performed using IAN cell lines as targets, for both autologous PBMC and MLR-derived effectors, in which the responding cell population was taken from the same individual used to generate the lines. The mean density of TFR expressed by the target cells was determined on the same population used for the cytotoxicity assay, during the 4 hr cytotoxicity incubation. The results shown in Fig. 4 represent a 50:1 E:T ratio, but they did not differ from other ratios used other than in magnitude (data not shown). PBMC showed limited, but distinct killing of most of the IAN lines, which was clearly titratable in all cases over a range of dilutions from 100:1–12·5:1. However, no relationship was observed between killing and TFR density. MLR-derived effectors used in these experiments showed higher levels of killing, but again no relationship to TFR density was detected. One of the main reasons for using similar target lines from a single individual was to eliminate possible differences between the cells, not directly related to the NK target structure, that may have affected susceptibility. However, these results may still be explained by such differences, so further experiments were performed to test this possibility.

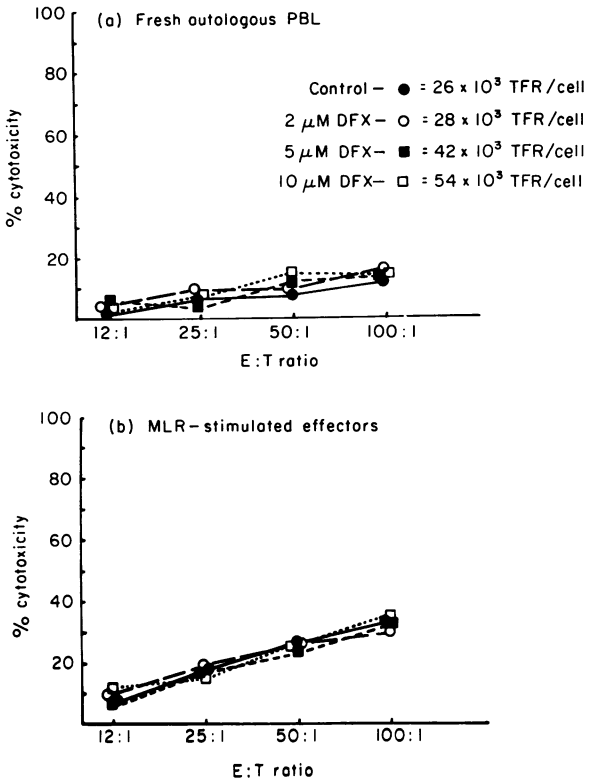

Figure 5. The relationship between transferrin receptor density and susceptibility to natural cytotoxicity using a single target cell line (IAN-6). The transferrin receptor density was artificially increased by incubation with various doses of the iron chelating agent desferrioxamine (DFX). The results shown depict one of three experiments, the results of which were consistent, and showed no changes in cytotoxicity related to TFR expression, either (a) for fresh autologous PBMC effectors or (b) MLR-derived effectors.

Table 2. Cold-target inhibition by the line IAN-6 of K562 killing

Ratio of IAN-6:K562	PBMC effectors		Day 6 MLR effectors	
	IAN-6	IAN-6+DFX	IAN-6	IAN-6+DFX
1:1	33·6	30·7	26·4	28·8
2:1	30·4	28·2	20·4	21·8
4:1	26·3	25·8	17·6	16·3
8:1	23·9	24·5	15·1	12·9
K562 only	37·8		42·9	

Values represent percentage cytotoxicity of the labelled K562 cells in assays incorporating four different concentrations of unlabelled inhibitors. The IAN cells were either treated with 10 μM desferrioxamine (giving a TFR density of 69×10^3 receptors per cell), or left untreated as a control (TFR density 36×10^3 receptors per cell). Progressive inhibition occurred with increasing numbers of IAN-6 cells, but there was no difference between the inhibitory potential of cells cultured with or without desferrioxamine (DFX).

The relationship between TFR density and NK susceptibility on a single cell line (IAN-6)

The mean TFR density of the cell line IAN-6 was 34,260 ± 4963 receptors per cell. This was very similar to figures achieved for MLR-activated lymphocytes in parallel experiments, so this line was comparable in terms of baseline TFR expression to activated lymphocytes in a peripheral response. IAN-6 cells showed a moderate susceptibility to killing by both PBMC and MLR-derived effectors. The density of TFR was increased on this line using the iron chelating agent desferrioxamine (DFX). Three experiments were performed, each using PBMC and MLR-derived effectors. A dose-dependent increase in TFR expression was observed on the treated target cells. In all cases, the maximum dose of DFX (10 μM) more than doubled the number of TFR compared to control cells. Using PBMC effectors, good cytotoxity titrations were obtained, though the actual level of killing was low. However, no difference in susceptibility was observed related to the density of transferrin receptors expressed by the targets, i.e. the cytotoxicity titrations were the same. Using MLR-derived effectors, higher levels of killing were obtained, but again no relationship to the density of transferrin receptors expressed by the target cells was observed (Fig. 5).

To confirm the lack of relationship observed between TFR expression and NK susceptibility, cold-target inhibition studies were performed using K562 cells as labelled targets. The E:T ratio was maintained constant at 50:1, and increasing concentrations of IAN-6 cells were added as an inhibitor of lysis. The IAN-6 cells were either treated with 10 μM DFX for 48 hr to increase the density of transferrin receptor expression, or left untreated as a control (Table 2). No difference was found between the inhibition by treated or untreated IAN-6, using either PBMC or MLR-derived effectors.

DISCUSSION

Several reports have suggested that NK cells may recognize the TFR as a target for killing (Vodinelich *et al.*, 1983; Newman *et al.*, 1984; Lazarus *et al.*, 1985). The present study sought to investigate whether such a mechanism could operate in the control of the peripheral immune response, since activated NK cells are reported to exist in significant levels at the site of such response (Tartof *et al.*, 1984).

Six EBV-transformed lymphoblastoid cell lines were produced from a single individual, and tested as targets against autologous effectors. The EBV-transformed lines showed a wide range of TFR expression, though those lines with lower expression showed a similar receptor density to that reported for activated lymphocytes (Bomford *et al.*, 1983). Two effector populations were tested: fresh peripheral blood mononuclear cells, and activated cells derived from a 6-day allogeneic MLR. In both of these populations, the cells responsible for killing were predominantly Leu-11$^+$ Leu-7$^+$ NK cells; this is in accord with the findings of Phillips & Lanier (1986). In the MLR-derived population, such cells are activated (Salmon *et al.*, 1985), apparently, by an IL-2-dependent process (Salmon & Bacon, 1986).

The six IAN cell lines used as targets were relatively resistant to unstimulated peripheral blood effectors, though a distinct but low level of titratable cytotoxicity was observed. Killing by

MLR-derived effectors was considerably more pronounced. No relationship was observed between the density of TFR expressed by the targets, and NK susceptibility using either effector population.

The main reason for using a number of similar lines was to minimize differences between them that may mask a relationship between target expression and killing. However, these preliminary experiments could not totally exclude such a possibility. For this reason the TFR density of a single line was increased using three separate doses of desferrioxamine (Mattia *et al.*, 1984); this treatment should not affect other surface structures. Again no increase in cytotoxicity was observed, even when the TFR density was doubled. To confirm this observation cold-target inhibition of K562 lysis was performed using the line IAN-6. Modulating the density of TFR expressed by the inhibitor line made no difference to its inhibitory potential. It is therefore highly unlikely that the proliferation of TFR-bearing lymphocytes is subject to control by this mechanism, as the transferrin receptor on lymphoid cell lines clearly does not act as a target for NK cell lysis.

Vodinelich *et al.* (1983) based their original theory on a degree of correlation between the number of TFR^+ cells in cell line cultures, and their susceptibility to natural cytotoxicity, though in fact several lines with very high TFR expression, such as HL60, are NK resistant. Furthermore, the TFR as a proliferation antigen, is co-expressed with a number of other fairly ubiquitous receptors for hormones and precursors, so such a relationship may well be indirect.

However, Vodinelich *et al.* (1983) also found that purified trypsin cleavage fragments of the TFR could partially inhibit killing of K562 cells. This evidence was far more convincing, but recently quite a range of virus-associated antigens have been shown to do the same (Biron & Welsh, 1982; de Martino *et al.*, 1985).

Newman *et al.* (1984) transected mouse L cells with human DNA, and achieved expression of human transferrin receptors by such cells. These transfected L cells produced a partial cold-target inhibition of K562 lysis, but interestingly mouse L cells without human transferrin receptors also produced significant inhibition. It is also noteworthy that this group could find no evidence for the TFR acting as an NK target in mice, yet if this were an important homeostatic mechanism in humans it would be unlikely to be so recently evolved. Lazarus & Baines (1985) studied the ability of K562 cells at different phases of growth to inhibit lysis of control K562. They showed that K562 cells express the highest density of TFR during log-phase growth, and that cells in this phase are most effective for competitive inhibition of NK killing. This is in accord with observations made in the present study, that cell lines are most effectively and reproducibly killed in log-phase growth. However, Lazarus & Baines (1985) suggested this as evidence that the TFR acts as a target for NK, but the data can be interpreted only as indicating that the actual target receptor is likely to be a structure preferentially associated with proliferation.

Bridges & Smith (1985) used a similar method of TFR modulation on K562 cells to that described in the present study. They increased and decreased expression of the TFR on K562 cells, and found that these changes made no difference either to NK susceptibility or to the efficacy of such cells in inhibiting lysis of control K562 targets. K562 erythroleukaemic cells have extremely high levels of TFR expression constitutively, and also very high NK susceptibility, so the results of Bridges & Smith (1985) may have reflected a saturated system in which an excess of receptors was present in all cases, regardless of experimental modulation. The present study, using lymphoid lines with relatively low expression of TFR, suggests that this was not the case, and that over a wide range of transferrin receptor expression there is no relationship whatsoever to NK susceptibility.

It is thus reasonable to conclude that the transferrin receptor does not act as a major NK target structure. The accumulation and production of activated NK cells at the sites of immune response (Tartof *et al.*, 1984; Salmon *et al.*, 1985) is in our view unlikely to play a homeostatic role. The effector to target ratios employed in this study were high, but yielded relatively low-order cytotoxicity. *In vivo*, effector cells are likely to be considerably less common, so natural cytotoxicity directed against activated lymphocytes would be very limited. Activated NK cells are perhaps more likely to be directed against virus-infected cells, or to act as producers of interferon-γ (Djeu, 1983).

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