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Allogeneic lymphocyte cytotoxicity (ALC) in rats: establishment of an *in vitro* assay, and direct evidence that cells with natural killer (NK) activity are involved in ALC

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SUMMARY

The evidence that NK cells can recognize and kill allogeneic lymphocytes has hitherto been based mainly on experiments in intact animals. Here we report results from an *in vitro* assay, showing allogeneic lymphocyte cytotoxicity in cell suspensions enriched for NK activity against tumour cells by Percoll gradient centrifugation of nylon-wool non-adherent cells. The addition of phytohaemagglutinin (PHA) to the NK-target cell cultures greatly enhanced the cytotoxic response against K562 and allogeneic, but not syngeneic, lymphocytes. The effector cells of ALC are present in the spleen of both euthymic and athymic nude rats, and to a lesser extent in the blood. ALC is augmented by interferon pretreatment of the effector cells, and by depleting the effector cell suspensions of all T cells and helper T cells with the monoclonal antibody MRC Ox19 and W3/25, respectively. Conversely, the activity was nearly abolished by depleting the cell suspensions of MRC Ox8$^+$ cells reacting with rat cytotoxic T cells and NK cells. Furthermore, removal of residual B cells (Ox12$^+$ cells) from the effector cells or attempts to block any putative antibody-dependent cellular cytotoxic mechanism *in vitro* with the monoclonal antibody Ox12 did not inhibit the NK activity against allogeneic lymphocytes nor against tumour cells. ALC *in vitro* did not discriminate between T and B or large and small lymphocyte targets. These characteristics of the ALC effector cells substantiate that they are present within the thymus-independent population of cells with NK activity, and are dependent on neither B cells nor immunoglobulin for their recognition and destruction of the target.

INTRODUCTION

In several species of animals, including the mouse, rat, sheep and pig, transfer of mature allogeneic lymphocytes into unprimed recipients often results in a prompt elimination of the cells (for review, see Rolstad & Ford, 1983). This elimination, referred to as allogeneic lymphocyte cytotoxicity (ALC, Rolstad & Ford, 1983), starts within 1 hr of i.v. infusion of the cells, takes place within the lymphoid tissue itself, and is enhanced in athymic nude rats, even when they are depleted of their B cells and immunoglobulins by treatment with an antibody to the μ-chain of rat Ig from birth (Tønnesen & Rolstad, 1983; Rolstad & Ford, 1983; Rolstad *et al.*, 1985; Fossum & Rolstad, 1986). These and other characteristics of the rejection mechanism categorize it into the broad group of 'natural' immune phenomena in which natural killer (NK) cells are important. In rats, ALC can be seen in congenic strain combinations of rats differing only at the MHC, suggesting that MHC-gene products are involved (McNeilage *et al.*, 1982). Furthermore, allorejection can occur of at least certain MHC incompatible cell types in the absence of mature T or B cells (Snell, 1976; Bordignon, Daley & Nakamura, 1985; Daley & Nakamura, 1984; Rolstad *et al.*, 1985). However, the idea of NK activity directed against normal allogeneic cells rests largely on experiments done in intact animals, and is in apparent contradiction to the many observations on the relative resistance of mature allogeneic cells to attack by NK cells *in vitro*. On the contrary, the cells most susceptible to NK lysis *in vitro* seem to be those lacking MHC antigens (Grönberg, Kiessling & Fiers, 1985; Kärre *et al.*, 1986).

This conflict has provoked the need for an *in vitro* assay for ALC, where the effector cells can be better characterized and the nature of the effector–target cell interaction more thoroughly studied.

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ALC, allogeneic lymphocyte cytotoxicity; CM, complete medium; FCS, fetal calf serum; IDC, interdigitating cells; IFN, interferon; Ig, immunoglobulin; mc ab, monoclonal antibody; MHC, major histocompatibility complex; NK cell, natural killer cell; NW, nylon-wool; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; PHA, phytohaemagglutinin; SC, spleen cells; TDL, thoracic duct lymphocytes.

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MATERIALS AND METHODS

Animals and operative procedures

Rats of the inbred strains PVG ($RT1^c$), DA ($RT1^a$) and AO ($RT1^u$) were bred in conventional animal houses. The nude mutant was bred into the PVG strain by at least five backcrosses, and was reared in isolators from breeding pairs originally purchased from OLAC, Bicester, Oxon, U.K. Thoracic duct drainage was by Gowans' technique (Ford, 1978). The thoracic duct lymphocytes (TDL) used as target cells were usually collected overnight at 4° in phosphate-buffered saline (PBS) containing 20 IU of Heparin (Novo, Copenhagen, Denmark) per ml.

Reagents and antibodies

The following mouse monoclonal antibodies (mc ab) against rat lymphoid cell subsets were a kind gift from Dr A. Williams, MRC, Cellular Immunology Unit, Oxford, U.K. (the cell surface molecules against which the mc ab are reacting are denoted in parentheses): W3/25 (CD4) reacting with T helper cells (Mason *et al.*, 1980), Ox8 (CD8) reacting with T suppressor/cytotoxic cells (Mason *et al.*, 1980), and NK cells (Reynolds *et al.*, 1981a), Ox19 (CD5) reacting with all post-thymic T cells and a subset of B cells (Dallman, Thomas & Green 1984) but not with rat NK cells (Woda *et al.*, 1984), and Ox12 reacting with the Ig kappa chain of allotype 1^a, present on approximately 95% of all B cells from the PVG strain (Hunt & Fowler, 1981; Gutman *et al.*, 1983). The goat anti-rat immunoglobulin (GARa/Ig) was purchased from Nordic Immunological Laboratories, (Tilburg, The Netherlands), phytohaemagglutinin (PHA) from Wellcome Diagnostics (Dartford, Kent, U.K.; lot K835730) and rat interferon from Stratech Scientific Ltd (London, U.K.; Cytimmune interferon reagents, cat. no. 40061).

Preparation of effector cells

Spleen cells (SC) or peripheral blood lymphocytes (PBL) were separated from erythrocytes by Isopaque–Ficoll centrifugation (density 1·077) and enriched for NK cells by incubation on columns of nylon wool (NW) (Leukopak, Fenwal Laboratories, Deerfield, IL) for 1 hr at 37° as detailed elsewhere (Timonen *et al.*, 1982; Reynolds *et al.*, 1981b). The medium used was RPMI-1640, with HEPES buffer (25 mM/l), 5% fetal calf serum (FCS) and a standard antibiotic anti-mycotic solution added (all reagents purchased from Gibco, Paisley, Renfrewshire, U.K.). This will later be referred to as complete medium (CM). In some experiments NK cells were further enriched by separation on a four-step discontinuous Percoll density gradient (Percoll purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden). For details of the procedure, see Timonen *et al.*, (1982), Reynolds *et al.*, (1981a, b) and Rolstad, Herberman & Reynolds (1986). The Percoll solution was corrected to 285 mosm/l with a PBS solution with salt concentration 10 times the physiological value (Gibco), and CM to 290 mosm/l with sterile water. Usually, the gradients were composed so that densities relative to water were 1·053 for the upper fraction, and 1·065, 1·075 and 1·082 for the subsequent three fractions. The separated effector cells were stored overnight at 4° in CM before being used for the assay.

In vitro *ALC and NK assay*

This is in essence a standard 4-hr NK assay. The target cells were either the human erythroleukaemic cell line K562 (kind gift from Dr Reynolds, National Cancer Institute, Frederick, MD) or TDL, separated from erythrocytes on Isopaque–Ficoll (density 1·09). They were labelled with $Na_2{}^{51}CrO_4$, (Amersham International, Amersham, Bucks, U.K.) at 200 μCi (approximately 8 MBq)/ml cells (K562) or 300 μCi (approximately 12 MBq)/ml cells (TDL) at the concentration of $5\text{-}10 \times 10^6$ cells/ml for 1 hr at 37°. The cells were washed three times in PBS + 2% FCS, and between the second and third wash incubated in about 40 ml PBS-FCS for approximately 30 min at room temperature. This incubation step reduced the spontaneous release from 40–50% of total c.p.m. in the cells down to 15–30%.

Effector and target cells were incubated in U-shaped Costar microtitre plates in a volume of 200 μl (Costar, Cambridge, MA; cat. no. 3799) at 10^4 targets/well, in CM for 4 hr at 37° in 5% CO_2. When PHA was added, the final dilution was 1/100 of the standard preparation. The supernatants were harvested with a Titertek harvesting system (Skatron, Lierbyen, Norway) removing approximately 85% of the supernatant. The percentage experimental ^{51}Cr release (exp.) was calculated according to the formula:

$$\text{exp.} = \frac{x - \text{SR}}{\text{T} - \text{SR}}$$

where x was the radioactivity in the test sample, SR was the amount of ^{51}Cr released from the target cells alone, and T was the total counts per minute (c.p.m.) in the target cells. With TDL as target cells, the total c.p.m. in 10^4 cells was about ten times the background count in the counter (i.e. 500 c.p.m.), whereas the T value for K562 was about 100 times the background (5000–10,000 c.p.m.). The results are presented as median values from triplicate assays for each effector:target cell ratio.

Interferon pretreatment

Interferon pretreatment of the effector cells was by incubation for 3 hr at 37° in CM, and with 800–1000 IU of rat interferon added to 10^7 cells per ml. The cells were washed once before plating.

Selection of effector lymphoid cell subsets by means of monoclonal antibodies and magnetic microspheres

NW-passed SC from PVG rats were incubated with the monoclonal antibodies (undiluted tissue culture supernatants containing 5–50 μg of protein/ml) at saturating conditions for 30 min on ice, washed once in PBS-FCS, and suspended in a solution of microspheres (Dynospheres M-450, Dynal A/S, Oslo, Norway) coated with a rabbit anti-mouse Ig (kind gift from Dr Nustad, Det Norske Radiumhospital, Oslo, Norway). For details of the coating procedure, see Nustad *et al.* (1986) and Lea *et al.* (1985). The microsphere to cell ratio was approximately 5:1, and the cells were incubated for 30 min at 4° in about 5 ml of CM with occasional gentle stirring of the cells. The cells rosetting with the microspheres were then removed from the non-rosetting cells with a magnet. For selection of $W3/25^-$ and $Ox19^-$ cells, the starting cell suspension containing 90×10^6 cells yielded from 14×10^6 to 28×10^6 negatively selected cells with less than 1% contamination of the positive cells. For Ox8 cells the starting population was 35×10^6–40×10^6 cells, yielding approximately 15×10^6 cells with less than 10% of $Ox8^+$ cells. The removal of $Ox12^+$ cells by the magnet beads reduced the frequency of $Ox12^+$ cells to $< 1\%$, but since about 90% of the B

cells had already been removed by the NW separation, this treatment had only a modest effect on cell yield.

Experimental design for testing the putative blocking effect of anti-rat immunoglobulin on NK activity and ADCC

Between 10×10^6 and 40×10^6 effector cells per ml of RPMI (NW-separated and Percoll gradient-enriched PVG spleen NK cells) were preincubated with antibody (GARaIg or MRC Ox12) for 1 hr at 37° and washed once before plating. For GARaIg the final concentration was 1/20 of the standard rehydrated specimen. For Ox12, the dilution was 1/100 of the ascites fluid, containing 22·5 mg protein/ml fluid, and with a titre against B cells of 1/10,000.

In order to avoid any ADCC of the anti-rat immunoglobulin itself through the binding to target B cells, we used here as target cells very early collections of TDL (0–2 hr) containing >90% T cells (=T−TDL).

To the cultures of antibody-treated effector cells and target cells was also added the same anti-immunoglobulin at a final dilution of 1/100. Also here, PHA was added to all cultures at a final dilution of 1/100.

In the ADCC assay the anti-target antibody (a strong DA anti-PVG antiserum raised by two skin grafts and with a cytotoxic titre against PVG-TDL of 1/256) was added directly to the effector target cell cultures at a final dilution of 1/20. Blocking with Ox12 was performed as in the NK assay, except that no PHA was added.

RESULTS

Optimalizing the *in vitro* conditions for ALC

In previous *in vitro* experiments we consistently observed a selective cytotoxicity of PVG rnu SC against allogeneic AO bone-marrow cells, but high and variable spontaneous releases (SR) of ^{51}Cr from target lymphocytes precluded any firm statements to be made about natural cytotoxicity against lymphoid target cells (Rolstad & Benestad, 1984; Rolstad *et al.*, 1985). This high SR from lymphocytic target cells *in vitro* could be substantially reduced by incubating the cells for at least 30 min at room temperature after labelling, thus allowing loosely bound ^{51}Cr to be eluted from the cells. With this modification, SC from PVG rnu rats exhibited a significant and consistent cytotoxicity against AO-TDL in all of the six experiments done in this series (Fig. 1). Moreover, the cytotoxicity both against the allogeneic lymphocytes and against K562 could be greatly enhanced by adding small amounts of PHA to the cultures, while the activity against syngeneic TDL remained zero (Fig. 1). Although PHA may induce non-specific cytotoxic responses in other test systems, e.g. via a polyclonal activation of T cells, we believe that the mode of action of PHA in the present experiments was to enhance a cytotoxic mechanism already existing among the nude effector cells by optimalizing the contact between effector and target cells, as previously described by Möller (1965). The observations supporting this conclusion were as follows: (i) the alloreactivity of nude SC was present even in the absence of PHA, (ii) the enhancement by PHA took place with effector cells devoid of alloreactive T cells (nude effector cells), (iii) syngeneic target cells were not affected, and (iv) SC from DA rats showed no reactivity against lymphocytes from the allogeneic rat strains AO or PVG, even in the presence of PHA (data not shown). DA rats exhibit cytotoxic T-cell responses against cells from both these strains, but lack ALC against both (Rolstad, 1979).

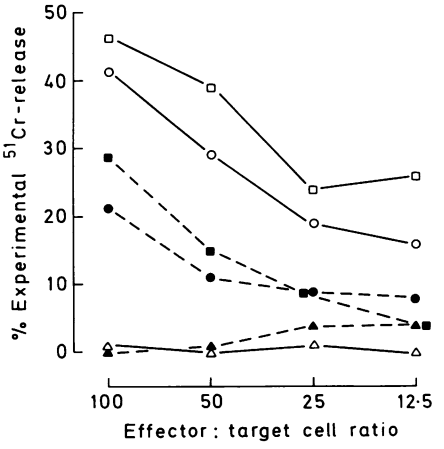

Figure 1. ALC *in vitro*: comparison between the cytolytic activity of PVG rnu spleen cells against PVG-TDL (△, ▲), AO-TDL (○, ●) and K562 (□, ■) in the presence (△, ○, □) or absence (▲, ●, ■) of PHA. Median values from four to eight experiments are presented.

Co-purification of NK cells and effector cells of ALC

Most of the NK activity in rats is associated with the morphologically identifiable large granular lymphocyte (LGL), which can be enriched from spleen and blood by Percoll density gradient centrifugation of nylon-wool (NW) passed cells (Timonen *et al.*, 1982; Reynolds *et al.*, 1981b). With PBL from euthymic PVG rats, the highest NK activity against K562 was found in the light-density cell fraction (F_2) of a Percoll density gradient, the fraction also containing the highest proportion of LGL (Fig. 2). Although low, it was only in this fraction that a significant NK activity against AO-TDL could be seen (Fig 2a). In contrast, with SC from rnu rats we obtained much higher ALC *in vitro* than with blood cells, but again NK activity against K562 and allogeneic TDL correlated with the presence of LGL in the different effector cell fractions (Fig. 2b).

Effect of interferon (IFN) pretreatment of the effector cells

Increased lysis of tumour target cells has been observed after IFN pretreatment of the effector cells (Reynolds *et al.*, 1982). This was also true for normal allogeneic lymphocytes as target cells; effector cells pretreated with a rat interferon exhibited significantly augmented *in vitro* ALC as well as NK activity against K562 (Fig. 3).

The ALC effector cell does not discriminate between lymphocyte subpopulations as target cells

AO-TDL depleted of T cells ($W3/25^+$ $Ox8^+$ and $Ox19^+$ cells) or of B cells ($Ox6^+$ and $Ox12^+$ cells) were tested as targets in ALC. No significant difference between the susceptibility of T- and B-cell depleted targets could be detected (Fig. 4a). A standard collection of TDL contains, in addition to small non-dividing lymphocytes, about 5–10% of rapidly dividing large lymphoid

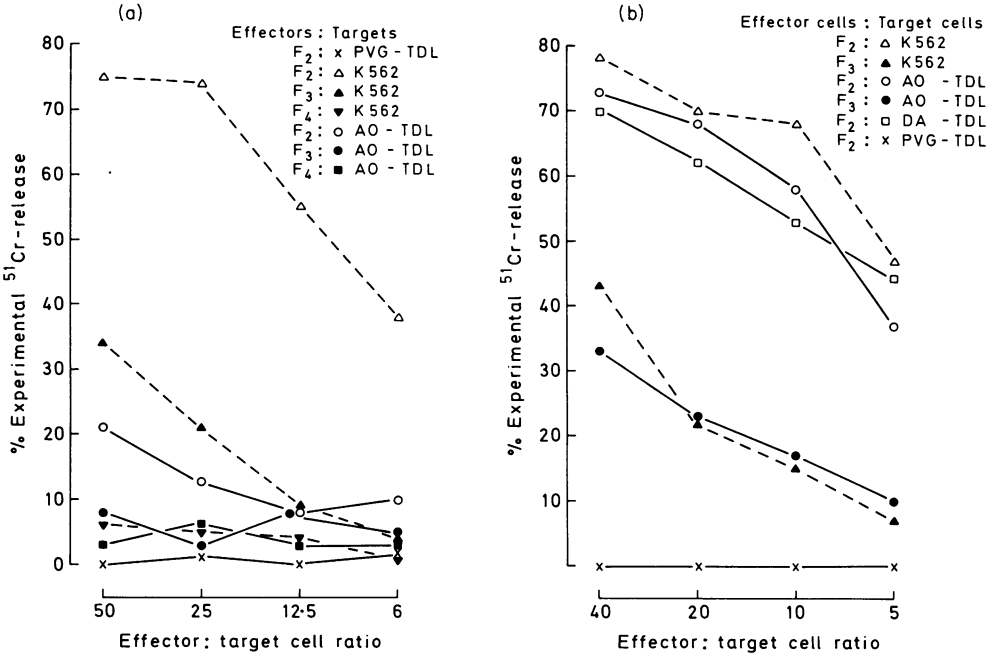

Figure 2. Co-purification of ALC and NK activity by NW separation and Percoll-density gradient centrifugation of effector cells. (a) PBL from PVG rats tested against PVG- or AO-TDL or against K562. F_2–F_4 = cell fractions 2–4 of NW-passed and Percoll-separated PBL containing 30–77% LGL (F_2), 1–6% LGL (F_3) and < 1% LGL (F_4). Values are medians from two to three experiments. (b) Spleen cells from PVG rnu rats, tested against AO (○, ●), DA (□) or PVG (×) TDL, and against K562 (△, ▲). F_2 54% LGL, F_3 7% LGL. Values are medians from two experiments, involving five nude donors in each experiments

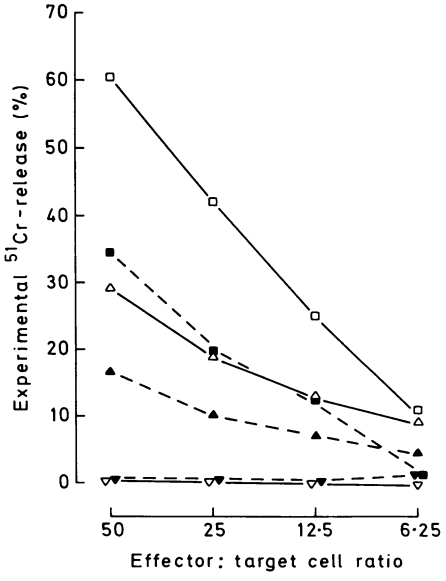

Figure 3. Effect of interferon pretreatment of effector cells on ALC and NK activity. Effector cells were NW-passed and Percoll-enriched NK cells from PVG spleen, either incubated with interferon (□, △, ▽) or control incubated cells (■, ▲, ▼). Target cells: K562 (□, ■), AO-TDL (△, ▲), PVG-TDL (▽, ▼). Results from one representative experiment are shown.

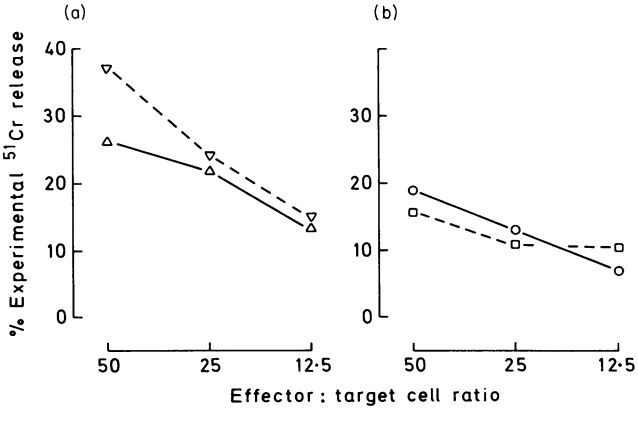

Figure 4. No differential sensitivity of target cell subpopulations to ALC *in vitro*. Effector cells: NW-passed and Percoll-enriched NK cells from PVG rats. (a) Target cells, AO-TDL, depleted of T cells by removal of $Ox19^+$, $W3/25^+$ and $Ox8^+$ cells (B cells, ▽) or depleted of B cells by removal of $Ox12^+$ and $Ox6^+$ cells (T cells, △) by magnet bead separation. (b) AO-TDL depleted of large dividing cells by Percoll gradient fractionation. Percoll F_3 contained < 1% large lymphocytes (○), whereas F_2 contained approximately 20% large lymphocytes (□). Results from one typical experiment are shown.

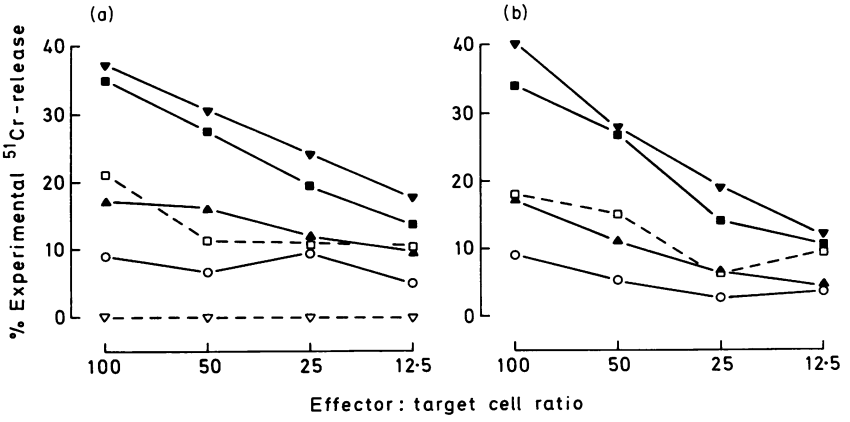

Figure 5. Phenotype of the effector cell in ALC and NK. Effector cells: NW-passed spleen cells from PVG rats, either untreated (□) or selectively depleted of Ox19$^+$ (▼, ▽), W3/25$^+$ (■), Ox12$^+$ (▲) or Ox8$^+$ (○) cells by magnet bead separation. Target cells: AO-(▼, ■, ▲, □, ○) or PVG-(▽) TDL (a) or K562 (b). The symbols represent medians from two experiments.

cells (Ford, 1978), and it could be argued that ALC *in vitro* was exclusively directed towards this subpopulation of TDL. This possibility was excluded in experiments in which we separated small from large lymphocytes by Percoll gradient centrifugation and then used them as targets. There was no difference in the sensitivity between the target cell fraction containing 20% large cells (F_2 cells) and the fraction containing less than 1% large cells (F_3 cells) (Fig. 4b), thus confirming our previous conclusion from *in vivo* experiments that ALC is directed towards small, non-dividing lymphocytes as well as lymphoblasts.

Phenotype of the effector cell of ALC

In rats, NK cells are characterized by the absence of cell surface markers characteristic of B cells and post-thymic T cells, except for the presence of the marker Ox8 which is shared with cytotoxic T cells (Reynolds *et al.*, 1981a; Woda *et al.*, 1984). In experiments in which we tested the NK activity and ALC of PVG-SC after removal of cells defined by the mc ab Ox19 (all T cells but not NK cells), W3/25 (T-helper cells), Ox12 (all B cells expressing the K chain) or Ox8 (T-cytotoxic cells and NK cells), a full concordance between the two systems could be observed, in that both effector cells apparently lacked the Ox19, W3/25 and Ox12 marker but expressed the Ox8 marker (Fig. 5).

Failure to inhibit ALC *in vitro* with an anti-rat immunoglobulin

The preceding data came close to excluding antibody-dependent mechanisms in the NK-mediated killing of allogeneic lymphocytes. However, it could be argued that the effector cells carried minute amounts of cytophilic antibody on their surface, or that small numbers of B cells escaping the nylon-wool or magnet bead separation were present among the effector cells, and that these cells secreted small amounts of anti-target cell antibodies into the medium, sufficient to initiate an antibody-dependent cellular cytotoxic mechanism (ADCC). In order to block effectively putative ADCC activity in our cultures, we preincubated our effector cells with anti-rat immunoglobulin, and also added the anti-Ig directly to the cultures of NK cells and target cells. In the first set of experiments we used the MRC Ox12 monoclonal antibody binding to the K chain of rat Ig, present on approximately 95% of all B cells. This antibody effectively inhibited the ADCC activity of DA-SC (in themselves devoid of NK activity against PVG cells, Fig. 6b) plus a strong DA anti-PVG alloantibody against PVG T-TDL as targets (Fig. 6b). In the same system, however, Ox12 did not block the NK activity of PVG-rnu SC against AO T-TDL or against K562 (Fig. 6a). Similar results were obtained when, instead of the mc ab Ox12, we used a polyvalent goat anti-rat immunoglobulin and euthymic PVG-SC as effector cells (data not shown).

DISCUSSION

Previous experiments on effector mechanisms of ALC have all pointed to an NK-like cell as the culprit; however, so far the evidence has been circumstantial and based on experiments done in intact animals by either depleting or enriching for NK activity (Heslop, McNeilage & Sengupta 1984; Rolstad *et al.*, 1985; Fossum & Rolstad, 1986). Here, we have directly demonstrated that SC, rich in NK activity, under appropriate experimental conditions also express ALC *in vitro*. Further similarities between ALC effector mechanisms and NK activity *in vitro* were striking: both were present among SC of normal as well as athymic nude rats, they both co-purified with the light-density cells by Percoll gradient centrifugation, both activities were enhanced by IFN pretreatment of the effector cells, and the effector cell surface phenotype indicated that neither post-thymic T cells nor B cells were involved.

This finding may seem somewhat surprising in the light of our previous observations that the cells phagocytosing the allogeneic lymphocytes within the lymphoid tissues have the morphological appearance of interdigitating cells (IDC) and not NK cells (Fossum & Rolstad, 1986). Apart from their bone-marrow origin, NK cells and IDC are not closely related cells, neither morphologically, phenotypically nor functionally (Fossum, 1984; Fossum & Ford, 1985). IDC display no NK activity *in vitro* (Fossum & Rolstad, 1986), but may have their prime function as antigen-presenting cells to the adaptive immune system. On the basis of these results, we have proposed as a working hypothesis that ALC *in vivo* is dependent on *both* NK cells and IDC: the NK cells discriminate between own and foreign, and kill the allogeneic cells, whereas IDC phagocytose

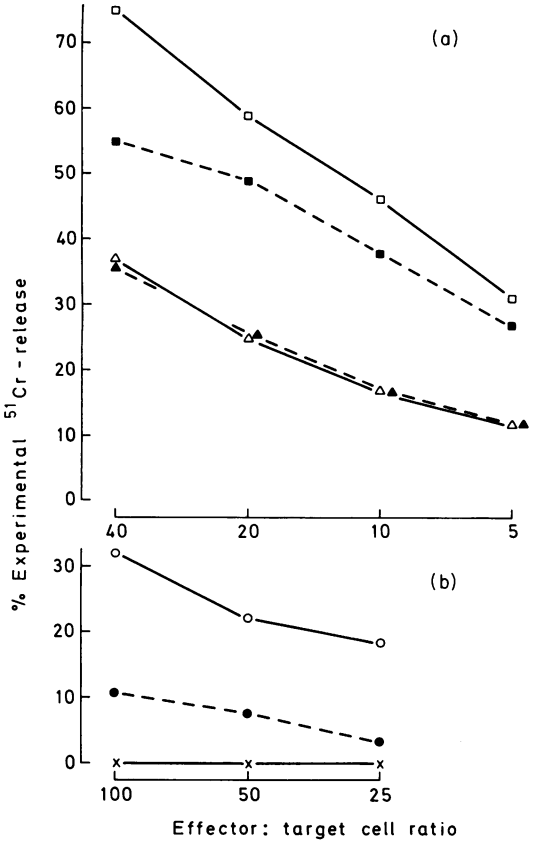

Figure 6. No blocking of ALC *in vitro* with the anti-rat immunoglobulin Ox12. (a) NK activity of PVG rnu SC (NW-passed and Percoll-separated, approximately 50% LGL) against K562 (□, ■), or AO-T-TDL (△, ▲); Ox12-pretreated effector cells, and Ox12 added to the cultures (■, ▲); control incubated effector cells (□, △). (b) ADCC of DA-NW-passed SC against PVG-T-TDL: cytotoxic activity of DA-SC alone (×), in the presence of a DA anti-PVG alloantiserum (○), in the presence of the alloantiserum +Ox12 (●).

the destroyed cells and possibly present their antigens to the adaptive immune system (Fossum & Rolstad, 1986). The close anatomical proximity of NK cells and IDC within the paracortex of the lymph nodes and the periarteriolar lymphoid sheaths of the spleen (Ward, Argilan & Reynolds 1983; Fossum & Rolstad, 1986) makes this type of collaboration quite feasible.

Although NK activity is operationally defined and NK cells are a heterogenous population of cells, including also some T cells (Klein, 1980; Lanier & Phillips, 1986), the presence of ALC in athymic rats cannot be attributed to polyclonally activated T cells. On the contrary, the present experimental model has shown great similarities between the thymus *in*dependent population of NK cells and the ALC-reactive cells, providing evidence to indicate that these two effector cell populations are closely related, if not identical. On this premise, the presently described experimental system provides a new tool in the characterization of effector–target cell interactions in thymus-independent NK phenomena.

Although our previous data from anti-μ treated animals came close to excluding naturally occurring antibodies as the specificity link (Rolstad *et al.*, 1985), it could still be argued that a minority of B cells escaping the depletion by anti-μ were secreting anti-MHC antibodies in sufficient quantities to incite an ADCC reaction. This possibility was effectively ruled out in the present experiments, where strong ALC could be observed in effector cell populations extensively depleted of B cells, and under conditions where a putative ADCC reaction was blocked by adding anti-rat Ig to the cultures. Although naturally occurring antibodies may play a role in other natural resistance phenomena, e.g. in the longer term rejection of H-2 incompatible marrow cells (Warner & Dennert, 1985), the immediate rejection of either parental bone marrow by F_1 hybrids (Daley & Nakamura, 1984; Bordignon *et al.*, 1985) or in allogeneic systems (Dennert, Anderson & Warner, 1986) may require allospecific mechanisms inherent in the NK cells themselves.

The question of how the NK cells recognize their targets is closely linked to the question of which cell lineage the NK cells belong to. A relationship to myeloid cells as well as to the T-cell system has been suggested. Since ALC is present in MHC-congenic rat strain combinations (McNeilage *et al.*, 1982; G.W. Butcher and A. Ager, manuscript in preparation), MHC-gene products are involved, but it is not known whether they operate at the level of the effector cells or the target cells, or both. The crucial role MHC gene products play in the restriction of T-cell recognition of antigens has led some investigators to believe that NK cells might represent some archaic form of T-cell recognition at the pre-thymic level, but, unlike cytotoxic T cells, NK cells exhibit no MHC-restriction but may be directed against allogeneic MHC products themselves. The presently described *in vitro* assay for MHC-directed NK activity should therefore provide a useful tool for characterizing the role of MHC gene products in natural immune phenomena.

In conclusion, the molecules involved in the effector–target cell interaction in ALC remain elusive. A role of naturally occurring antibodies, or of cytotoxic T cells, now seems highly implausible, but whether ALC makes use of already existing gene material coding for receptor specificities within the adaptive immune system, or whether a new allorecognition system is on its way to be uncovered, still remains to be resolved.

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