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Interleukin 2 enhances natural killing of varicella-zoster virus-infected targets

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SUMMARY

Preincubation of peripheral blood non-adherent mononuclear cells with purified or recombinant interleukin 2 (IL-2) significantly enhanced natural killer (NK) activity against uninfected and varicella-zoster virus (VZV)-infected targets, while antibody-dependent cellular cytotoxicity (ADCC) against VZV-infected targets was not increased. Preincubation of effector cells with IL-2 had no effect on conjugate formation, but lysis of both targets was increased in single cell assays. IL-2-enhanced NK against VZV-infected targets was independent of gamma-interferon (γ-IFN) production.

Keywords NK to varicella-zoster virus

INTRODUCTION

Peripheral blood mononuclear cells (PBMC) mediate natural killing (NK) and antibody-dependent cellular-cytotoxicity (ADCC) against varicella-zoster virus (VZV)-infected fibroblasts (Kamiya *et al.*, 1982; Ihara *et al.*, 1983; Babbage, Sigfusson & Souham, 1984).

Interferons are well known to stimulate NK activity (Trinchieri & Santoli, 1978). We previously reported that PBMC incubated with alpha-interferon (α-IFN) demonstrate enhanced NK activity but not ADCC, against VZV-infected targets (Ihara *et al.*, 1983). Interleukin 2 (IL-2) has also been shown to enhance NK activity of murine (Henney *et al.*, 1981) and human (Domzig, Stadler & Herberman, 1983) effector cells *in-vitro*, but the effect of IL-2 on cytotoxicity against virus-infected targets has only been examined for cytomegalovirus (Rook *et al.*, 1983). We report on the effects of IL-2 on NK and ADCC against VZV-infected targets. Only NK activity was enhanced by IL-2. The mechanism of enhancement involved increased lytic ability and did not appear to depend on gamma-interferon (γ-IFN) secretion.

MATERIALS AND METHODS

Interleukin 2 preparations. Purified IL-2 (Electro-Nucleonics Inc., Silver Spring, MD, USA) was prepared from supernatants of phytohemagglutinin-stimulated human peripheral blood leucocytes by several chromatographic steps. Recombinant IL-2 (rIL-2) was produced by *E. coli* transfected

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with an expression plasmid containing the IL-2-coding region cloned from human lymphocytes (Trinchieri *et al.*, 1984). The purity of this preparation was >99%, and the specific activity was 3–10×10^6 u/mg. The concentrations of the two IL-2 preparations were 470 u/ml and 1280 u/ml respectively in assays for ^{3}H/thymidine incorporation by IL-2 dependent CTLL-2, cloned murine cytotoxic T lymphocytes (Baker, Gillis & Smith, 1979).

Preparation of effector cells. Heparinized peripheral blood was obtained from healthy adults, and mononuclear cells were separated by Ficoll-Hypaque (Pharmacia, Piscataway, NY, USA) gradient centrifugation. The cells were allowed to incubate for 1 h at 37°C in fetal bovine serum (FBS) (Hyclone, Logan UT)-coated plastic petri dishes (Lux Scientific Corporation, Newbury, CA, USA) (Kumagai *et al.*, 1979). Nonadherent cells were washed twice with Hank's balanced solution (HBSS) (GIBCO, Grand Island, NY, USA) and resuspended in RPMI-1640 (GIBCO, Grand Island, NY, USA) with 10% FBS at a concentration 3×10^6/ml. Effector cells were preincubated with various doses of IL-2 at 37°C, washed twice with HBSS, and resuspended in RPMI-1640 with 10% FBS. In selected experiments, effector cells were depleted of NK cells by treatment with anti-Leu-11b (Becton Dickinson Mountain View, CA, USA) plus complement (C′) as previously described (Ito *et al.*, 1985). This monoclonal antibody, reacts with the Fc receptor on NK cells and polymorphonuclear leukocytes. Briefly, aliquots of 1×10^6 non-adherent cells in 100 μl of RPMI-1640 were incubated with 0·1 μg of anti-Leu-11b for 45 min at room temperature. The cells were washed once and then resuspended in 100 μl of baby rabbit C′ (Pel-Freeze, Rogers, AZ, USA) diluted 1:4 with RPMI-1640. After incubation for 60 min at room temperature on a rocker, the cells were washed once and resuspended in RPMI-1640 with 10% FBS.

NK and ADCC assays. NK and ADCC assays were performed as previously described (Kamiya *et al.*, 1982; Ihara *et al.*, 1983). Briefly, frozen uninfected and VZV-infected human foreskin fibroblasts (FS4 strain) were thawed rapidly, washed twice in HBSS with 5% FBS, and then labelled with 50 μCi of $Na_2{}^{51}CrO_4$ at 37°C (New England Nuclear, Boston, MA, USA). After 1 h incubation at 37°C the labelled target cells were washed twice with cold HBSS with 5% FBS and resuspended in RPMI-1640 with 10% FBS at a concentration of 5×10^4/ml. Aliquots of 0·1 ml of labelled targets were added to wells of round-bottomed microtitre plates. For ADCC assays, 10 μl aliquots of seropositive and seronegative human serum pools prepared as previously described (Kamiya *et al.*, 1982), were added to 5×10^3 target cells in 100 μl for 30 min at 4°C. The final serum dilution was 1:100 based on previous observations that near maximal ADCC was detected with this concentration of antibodies (Kamiya *et al.*, 1982). Aliquots of 0·1 ml of effector cells or media were then added. All determinations were done in triplicate. Microtitre plates were centrifuged at 100 *g* for 3 min and incubated for 18 h at 37°C in 5% CO_2. After incubation, the upper 100 μl of media were gently removed from each well, and the ^{51}Cr activity was determined by gamma scintillation counting. The per cent ^{51}Cr release was calculated with the following formula:

$$\frac{\text{ct/min experimental} - \text{ct/min spontaneous}}{\text{ct/min total} - \text{ct/min spontaneous}} \times 100$$

where ct/min total is the release occurring after the addition of 1% triton X-100 to target cells, ct/min spontaneous is the release from target cells incubated with media alone, and ct/min experimental is the release occurring in the presence of effector cells. Spontaneous release from target cells was less than 40% in 18 h. The percent ADCC was defined as the per cent ^{51}Cr release occurring in the presence of seropositive sera minus the per cent ^{51}Cr release occurring in the presence of seronegative sera.

Effect of addition of anti-IFN. A monoclonal antibody to γ-IFN (B133.3, Trinchieri *et al.*, 1984) was provided by Giorgio Trinchieri, Wistar Institute. This antibody had a neutralizing capacity of 32,400 iu/ml. The antibody was added to media containing IL-2, and the mixtures were incubated for 1 h at room temperature. Effector cells were then added, and the incubation was continued for 18 h. The final antibody dilution was 1:5 (neutralizing capacity of 6,480 iu/ml). At the end of the incubation period, the cells were centrifuged at 350 *g* for 10 min. Supernates were collected, stored at −70°C, and subsequently tested for their ability to inhibit the cytopathic effect of vesicular stomatitis virus in foreskin fibroblasts as previously described (Starr *et al.*, 1980). Interferon

concentrations were expressed in international units by comparison to results obtained with NIH γ-IFN standard Gg 23-901-530. The pelleted cells were washed twice with HBSS, resuspended in RPMI-1640, and then tested for cytotoxic activity.

Immunofluorescent staining. Effector cells treated with anti-Leu-11b plus C′ and untreated cells were stained with monoclonal antibody B73.1 which reacts with the Fc-IgG receptor on NK cells and neutrophils (Perussia *et al.*, 1983). Aliquots of 1×10^6 cells were incubated with B73.1 at a final dilution of 1:200 for 30 min at 4°C, washed twice, and incubated with fluorescein conjugated goat $F(ab')_2$ antimouse Ig (Cappel Laboratories, West Chester, PA, USA) for 30 min at 4°C. The cells were washed four times with phosphate buffered saline (PBS), resuspended in PBS, and then analysed for percent fluorescence using a cytofluorograph (Ortho Diagnostic Systems, Westwood, MA, USA) as previously described (Perussia *et al.*, 1983).

Single cell assay. Single cell assays were performed using a modification of a previously described technique (Neville, Grimm & Bonavida, 1980). The targets used were the same as for ^{51}Cr release assays. Aliquots of 1×10^5 target cells in 100 μl of RPMI-1640 with 10% FBS were mixed with an equal number of effector cells in 15 ml conical tubes (No. 2095, Falcon, Oxnard, CA, USA). After 5 min incubation at 37°C the tubes were centrifuged at 50 *g* for 5 min at room temperature. The supernatant was removed and 50 μl of prewarmed RPMI-1640 was added. The cell pellets were resuspended by gentle pipetting. Ten microlitres of cell suspension were removed, and examined in a hemocytometer. Fifty μl of molten (39–40°C) 1% agarose (type 1, Sigma, St Louis, MO, USA) in RPMI-1640 were then added to the remaining cell suspension. The mixture was quickly spread over 1 × 3 inch × 1·2 mm glass slides (Fisher Scientific Co., Pittsburgh, PA, USA) previously coated with 1% agar (Sigma, St Louis, MO, USA). The slides were placed in glass petri dishes which were filled with RPMI-1640 with 10% FBS and then incubated for 18 h at 37°C in 5% CO_2. Target cells were also incubated without effector cells. After incubation, slides were stained with 1% trypan blue (Sigma, St Louis, MO, USA) for 5 min, washed three times with PBS, fixed for 5 min in 0·5% methanol (formaldehyde) and examined by light microscopy.

The per cent conjugates formed and per cent target cell lysed were calculated as follows:

$$\text{Per cent conjugates} = \frac{\text{No. of effector cells forming conjugates}}{\text{750 effector cells}} \times 100$$

$$\text{Per cent target cells lysed} = \frac{\text{No. of conjugates with dead targets}}{\text{100 conjugates}} \times 100$$

Lysis attributable to effector cells was defined as lysis in the presence of effector cells minus lysis in the absence of effector cells.

RESULTS

Effect of purified IL 2 on NK and ADCC

In preliminary experiments NK activity against uninfected and VZV-infected targets was augmented when effector cells were preincubated with increasing doses of purified IL-2 (Fig. 1). Subsequently, effector cells were preincubated with 32 u/ml of purified IL-2 or media alone for 18 h and tested for NK and ADCC activity. Preincubation of effector cells with IL-2 significantly enhanced cytotoxicity against both uninfected and VZV-infected targets (Table 1). The magnitude of IL-2-stimulated NK was greater against VZV-infected than against uninfected targets. With effector cells preincubated in media, cytotoxicity against VZV-infected targets was greatly enhanced by the presence of seropositive sera (Table 1), as previously reported (Kamiya *et al.*, 1982). In contrast, IL-2-stimulated cytotoxicity against VZV-infected targets, which was already quite high in the presence of seronegative sera, was only moderately enhanced by the presence of seropositive sera. Thus ADCC, defined as the amount of cytotoxicity attributable to the presence of antibody, was lower for effector cells preincubated with IL-2 (13·9%) than for effector cells incubated in media alone (33·5%).

Kinetics of IL-2 stimulated NK

When effector cells were preincubated with 16 u/ml of IL-2 for 18 h and then tested for NK cytotoxicity most of the lysis of uninfected and VZV-infected targets occurred during the first 4 h of

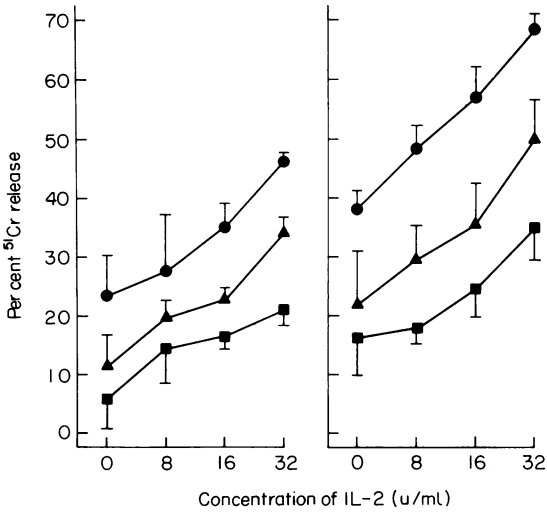

Fig. 1. IL-2 induced enhancement of NK activity. The effector cells were preincubated with different concentrations of purified IL-2 for 18 h. Targets were uninfected (left) or VZV-infected (right). Three different effector/target ratios were used. (●) 50:1; (▲) 25:1; (■) 12·5:1. Assay time was 18 h. Each data point represents the mean ± s.d. for at least two separate experiments.

Table 1. Effect of preincubation of effector cells with IL-2 on NK and ADCC*

Concentration of purified IL-2 in incubation media (u/ml)	Mean per cent ^{51}Cr release ± s.d. from target cells (no. of experiments)			
			VZV-Infected targets in the presence of serum	
	uninfected	VZV-infected	seronegative	seropositive
0	11·4 ± 5·0 (5)	23·7 ± 10·0 (8)	20·1 ± 9·0 (8)	53·6 ± 12·1 (8)
32	33·9 ± 16·2 (5)‡	46·3 ± 15·0 (8)§	42·9 ± 12·2 (8)§	56·8 ± 12·3 (8)†

* Effector cells were preincubated with IL-2 or media alone for 18 h, washed, and then added to target cells at an E:T ratio of 25:1. The assay time was 18 h.
† $P < 0.05$ compared to incubation with media alone (paired t-test).
‡ $P < 0.02$ compared to incubation with media alone (paired t-test).
§ $P < 0.001$ compared to incubation with media alone (paired t-test).

the assay, with some additional killing detected after 8 and 18 h (Fig. 2a). In contrast, with unstimulated effector cells little cytotoxicity was detected at 4 h, and most of the killing occurred between 8 and 18 h as previously reported (Baker, Gillis & Smith, 1979). Effector cells preincubated with 32 u/ml of IL-2 for as little as 4 h showed increased cytotoxicity against both targets compared to effector cells incubated with media alone (Fig. 2b); however, the magnitude of NK, particularly against uninfected targets, was higher when effector cells were preincubated with IL-2 for 18 h.

Effect of purified and recombinant IL-2 on NK

Effector cells preincubated with purified or recombinant IL-2 for 18 h lysed both uninfected and VZV-infected targets in a dose-dependent fashion, and the magnitude of killing was equivalent with

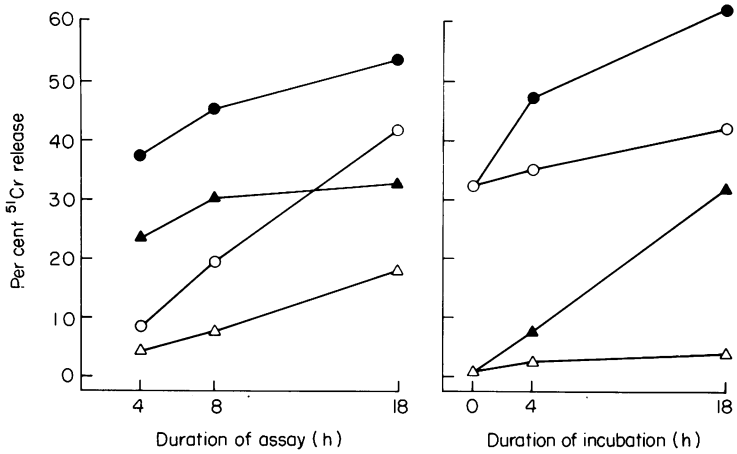

Fig. 2. Kinetics of enhancement of NK activity by IL-2. Target cells were uninfected (▲, △) or VZV-infected (●, ○). Effector cells were preincubated with purified IL-2 (●, ▲) or media (○, △) for 18 h, and tested for ability to mediate NK in 4, 8, 18 h assays (left). Effector cells were preincubated with IL-2 (●, ▲) or media (○, △) for 4 or 18 h and tested for NK in 18 h assays (right). E:T ratio was 25:1 in both experiments.

identical doses of purified and rIL-2 (results not shown). In all subsequent experiments 32 u/ml of rIL-2 was used.

Lack of involvement of γ-IFN in IL-2 stimulated NK activity

The presence of monoclonal antibody to γ-IFN during preincubation of effector cells with rIL-2, had little or no effect on IL-2 stimulated cytotoxicity (Table 2). The antibody was added in amounts sufficient to neutralize IFN produced by IL-2-stimulated effectors. In contrast, enhancement of NK activity by 100 iu/ml of recombinant γ-IFN was completely blocked by addition of this antibody during the preincubation period (data not shown). IFN concentrations in supernates of cultures of effector cells preincubated with rIL-2 were variable (0 to 512 iu/ml) among the different donors. IFN was not detected in duplicate cultures containing antibody to γ-IFN.

Table 2. The role of γ-IFN in IL-2 stimulated NK activity

	Cytotoxicity against VZV-infected targets mediated by effector cells incubated with*				IFN concentrations (iu/ml) in cultures of effector cells incubated with†	
Donor	media alone	anti-γ-IFN‡	rIL-2§	rIL-2+ anti-γ-IFN‡	rIL-2	rIL2+anti-γ-IFN
1	19·6	16·6	39·5	33·3	<2	<2
2	8·1	4·1	25·2	21·4	8	<2
3	19·9	13·2	41·0	39·8	512	<2

* Effector cells were preincubated as indicated for 18 h, washed and then added to target cells at an E:T ratio of 25:1. The assay time was 18 h.

† Interferon concentrations were determined for supernatants collected at the end of the preincubation period.

‡ The monoclonal antibody to γ-IFN (B133.3) was used at a final dilution of 1:5 which corresponded to a neutralizing capacity of 6,480 iu/ml.

§ Recombinant IL-2 was added at a concentration of 32 u/ml.

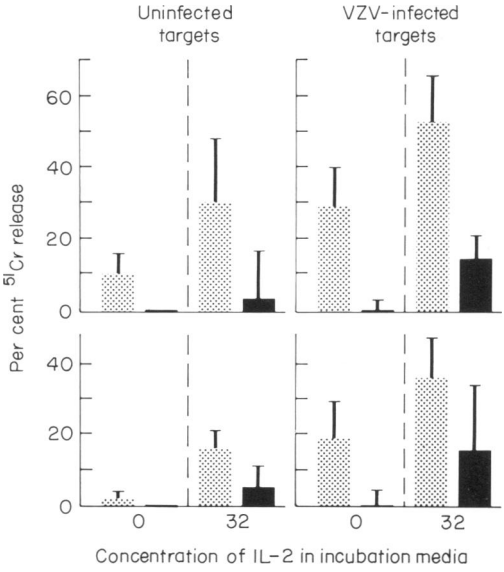

Fig. 3. Effect of treatment of effector cells with anti-Leu-11b plus C′. Effector cells were treated with anti-Leu-11b plus C′ (■) or C′ alone (■) before (upper panels) or after (lower panels) preincubation with media or 32 u/ml of IL-2 for 18 h. The data represent mean per cent ^{51}Cr release ± s.d. for three different experiments.

Table 3. Effect of incubation of effector cells with IL-2 on binding and lysis in single cell assays*

Concentration of IL-2 in incubation media (u/ml)	Mean per cent conjugate formation and lysis ± s.d. with different targets (no. of experiments)			
	uninfected		VZV-infected	
	conjugates	lysis	conjugates	lysis
0	1·9 ± 0·5 (6)	2·0 ± 2·5 (4)	4·2 ± 0·9 (6)	3·8 ± 5·3 (5)
32	2·9 ± 1·3 (6)	4·3 ± 1·9 (4)†	4·1 ± 1·2 (6)	9·4 ± 6·1 (5)†

* Effector cells were preincubated with media or rIL 2 for 18 h, washed, and then tested for conjugate formation and lysis in single cell assays as described in the Methods.

† $P < 0.01$ compared to incubation with media alone (paired *t*-test).

Effect of treatment of effector cells with anti-Leu-11b plus C′

To determine whether NK cells mediate Il-2 enhanced cytotoxicity, effector cells were treated with anti-Leu-11b plus C′, or C′ alone, before or after preincubation in the presence or absence of rIL-2. Treatment of effector cells with C′ alone before or after preincubation with media or rIL-2 had little or no effect on cytotoxicity compared to no treatment (results not shown). Treatment of effector cells with anti-Leu-11b plus C′ before or after preincubation in media or rIL-2 reduced the percentage of B73.1 positive cells from 4·0–7·4% to 1·1–1·5% (results not shown). Such treatment of effector cells before or after preincubation in media alone almost completely eliminated cytotoxicity against both targets (Fig. 3): treatment of effector cells before or after preincubation with rIL-2 also

resulted in markedly reduced killing of both targets, but the effect was less complete than that observed with unstimulated effector cells.

Effect of IL 2 in single cell assays

Effector cells were preincubated with 32 u/ml of rIL-2 or media alone for 18 h and tested for conjugate formation and lytic ability in single cell assays (Table 3). Per cent conjugate formation with uninfected or VZV-infected targets was unchanged by preincubation of effector cells with rIL-2, but in single cell assays lysis of both uninfected and VZV-infected targets was significantly increased.

DISCUSSION

Preincubation of non-adherent PBMC with purified or recombinant IL-2 significantly enhanced NK activity against uninfected and VZV-infected targets. It has previously been shown that both purified and recombinant IL-2 can augment NK activity against tumour cell targets (Trinchieri *et al.*, 1984; Weigent, Stanton & Johnson, 1983; Miyasaka *et al.*, 1984; Svedersky *et al.*, 1984), but the effect of IL-2 on NK activity against virus-infected targets remains largely undefined. Rook *et al.* (1983) reported that preincubation of effector cells with purified IL-2 enhanced CMV-specific cytotoxicity in patients with acquired immunodeficiency syndrome (AIDS), but not in normal adults. Our results in normal adults were similar in that after preincubation in IL-2, killing of uninfected and VZV-infected fibroblasts rose to about the same extent (Table 1), so that there was no net increase in the killing of VZV-infected targets. We have also obtained comparable results with CMV-infected targets (Bandyopadhyay *et al.* unpublished observations).

In contrast to the effect on NK, ADCC was decreased after preincubation of effector cells in IL-2. ADCC was lower because the presence of antibodies to VZV did not enhance IL-2-stimulated cytotoxicity to the extent that their presence enhanced cytotoxicity by unstimulated effector cells. Previously, Kimber *et al.* (1984) reported that ADCC against anti-D sensitized human O Rh (D) positive erythrocytes was enhanced by IL-2, and Shaw *et al.* (1985) reported that IL-2 augmented ADCC against osteogenic sarcoma cells. In both of these reports the targets used were relatively resistant to NK, unlike the fibroblasts used in our study, which may explain the discordant results. The magnitude of IL-2-stimulated NK against VZV-infected targets may approach the maximal possible lysis by either NK or ADCC mechanisms at the effector/target ratios used.

IL-2 has been shown to induce γ-IFN secretion by peripheral blood leucocytes (Kawase *et al.*, 1983). Whether γ-IFN contributes to enhancement of NK activity by IL-2 is controversial. Weigent, Stanton and Johnson (1983) reported that the ability of IL-2 to stimulate NK activity was completely blocked by the addition of antiserum to γ-IFN during the preincubation period. However, in several recent investigations (Trinchieri *et al.*, 1984; Lanier *et al.*, 1985; Rook *et al.*, 1985) the addition of anti-γ-IFN antibodies had little or no effect on IL-2 stimulated-NK activity. Differences among these studies may be attributable to the different antibody preparations or assay conditions. In our experiments, the magnitude of IL-2 stimulated cytotoxicity was independent of the IFN concentrations detected in supernates of cultures preincubated with rIL-2, and the addition of a monoclonal antibody to γ-IFN, which completely neutralized antiviral activity, did not inhibit the enhancement of NK activity by IL-2. These results suggest that γ-IFN is not required for IL-2 mediated enhancement of NK activity against VZV-infected targets.

The effects of IL-2 on binding of effector cells to virus-infected target cells and lysis in single cell assays have not been previously reported. We found that preincubation of effector cells with IL-2 had no effect on binding to uninfected and VZV-infected targets. In contrast, lysis of both targets was significantly increased in single cell assays, suggesting that the major effect of IL-2 is on the lytic capacity of NK cells.

To identify the cells mediating IL-2 enhanced cytotoxicity, NK cells were depleted by antibody plus C′ mediated lysis. NK activity against K562 was completely abolished by treatment with anti-Leu-11b, a monoclonal antibody which reacts with the IgG Fc receptor on NK cells and neutrophils, plus C′ (Itoh *et al.*, 1985). As previously reported (Ito *et al.*, 1985), depletion with anti-Leu-11b plus C′ almost completely eliminated NK activity of unstimulated mononuclear cells

against uninfected and VZV-infected targets. Treatment with anti-Leu-11b plus C′ also greatly reduced IL-2-stimulated cytotoxicity against both targets. In these depletion experiments a small percentage (from 1·1% to 1·5%) of B73.1 positive cells remained after treatment with anti-Leu-11b plus C′. These remaining NK cells, activated by preincubation with IL-2, may account for the residual cytotoxic activity detected. Additional experiments are in progress to identify further the effector cell(s) contributing to IL-2 stimulated killing of virus-infected targets.

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