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Lysis of varicella zoster virus infected cells by lymphocytes from normal humans and immunosuppressed pediatric leukaemic patients

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SUMMARY

Varicella zoster virus (VZV) is an important cause of morbidity and mortality in immunosuppressed children but little is known of the cellular mechanisms of VZV immunity. We therefore developed a clinically applicable system to study responses to VZV infected cells. Fresh blood mononuclear cells (MNC) from VZV immune donors killed VZV infected fibroblasts in an 18 h ^{51}Cr release assay. The specificity for virus was confirmed by cold target inhibition. An enhancing role for HLA matching was demonstrated using targets mismatched for HLA, and blocking by antibodies to HLA framework and T cell subsets. Cytotoxicity was not blocked with anti-Ia or anti-VZV antibodies. Killing of VZV infected target cells was reduced in seven out of nine VZV antibody positive patients in remission who were receiving maintenance treatment for acute lymphocytic leukaemia. Three of these patients had normal lymphocyte proliferative responses to VZV. Of the two patients with normal cytotoxic responses to VZV, one had reduced proliferation. It therefore appears that presence of VZV antibody, T cell proliferative responses, and cytotoxicity are independently variable. Cytotoxicity may be more susceptible to immunosuppression than either antibody or T cell proliferation.

Keywords Herpes virus cytotoxicity immunosuppression varicella leukaemia

INTRODUCTION

T cell-mediated immunity is normally stimulated following herpes simplex virus (Yasukawa, Shiroguchi & Kabayashi, 1983), cytomegalovirus (Quinnan *et al.*, 1981) and Epstein–Barr virus (Slovin, Schooley & Thorley-Lawson 1983) infections. Little attention has been paid to another Herpes virus, Varicella zoster virus (VZV).

Negative tests for VZV antibody can predict patients who are susceptible to a primary varicella infection but persistence of antibody does not ensure that reactivation of VZV infection, either as recurrent primary disease or more conmmonly as herpes zoster, will not occur in the immunosuppressed patient. Many investigators have attempted to correlate the patients' *in vitro* lymphocyte responses to VZV with subsequent reactivation of VZV (Mazur & Whitley, 1978; Patel *et al.*, 1979; Hayes *et al.*, 1982; Meyers, Flournoy & Thomas, 1980) and our own results indicated that leukaemic patients' T cell proliferation to VZV declined during treatment more often than the serum antibody titre to VZV (Giller *et al.*, 1983). Overall, however, the correlations between

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lymphocyte proliferation, antibody titre and VZV reactivation are poor. A likely explanation is that existing laboratory tests for VZV immunity ignore specific cellular cytotoxicity.

To address these questions we tested mononuclear cells from healthy controls and patients receiving leukaemia remission treatment for cell-mediated cytotoxicity against VZV infected targets.

MATERIALS AND METHODS

Subjects. Patients receiving remission maintenance treatment for acute lymphocytic leukaemia were recruited from the Pediatric Oncology Clinic at Denver Children's Hospital. Samples of their bone marrow and peripheral blood were obtained in the course of routine treatment monitoring, subject to informed consent. Bone marrow and peripheral blood were also drawn from consenting healthy adults.

Fluorescence assay for antibody to VZV membrane antigen (FAMA). Serum was tested for antibody to VZV antigen titres by a standard method (Zaia & Oxman, 1977). Titres of 1 : 2 or greater are defined as positive.

Preparation of effector cells. Blood was defibrinated and the serum saved. MNC were separated on a Ficoll/Hypaque density gradient and washed twice in Hanks' balanced salt solution (HBSS). Percoll gradient centrifugation to prepare effector cells enriched for NK cells was as described by Timonen & Saksela (1980).

HLA typing. Subjects were HLA typed using fresh blood MNC and NIH basic trays.

VZV and antigen preparation. Cell free VZV (strain Cp5, and 262 from the Center for Disease Control) was prepared in human lung fibroblasts between passages 13 and 21. Infected cells were harvested at 75% cytopathic effect, sonicated in a Braun Sonic 1510 sonicator (100 W, 0·25 of maximum power) for 40 s at 4°C in 5–10 ml of media, and clarified at 630*g* for 5 min at 4°C. The supernatant was brought to 7% sorbitol by adding 50% sorbitol, and the VZV was stored at −70°C (at $5{\cdot}2 \times 10^5$ PFU/ml). The virus preparations routinely used to infect target cells for the cytotoxicity assay were suspensions of frozen–thawed monolayers of bone marrow derived fibroblast which had previously been infected with VZV at a multiplicity of infection (MOI) of 0·1 and scraped down into 1 ml of Dulbecco's MEM with 10% fetal calf serum (FCS) and 7% sorbitol when marked cytopathic effect (CPE) was observed. These preparations were frozen in 0·2 ml/vial at −70°C for future use.

VZV antigen was prepared by glycine extraction of human embryonic lung fibroblast cultures showing greater than 75% CPE, by the method of Zaia, Leary & Levin (1978). Control antigen was extracted in a similar fashion from uninfected cell cultures.

Assay of lymphocyte proliferation to VZV. MNC at 10^6/ml in RPMI 1640 with 10% fresh autologous serum were cultured in 0·2 ml volumes in triplicate in U shaped microtitre wells (Linbro, No. 76-013-05) with VZV antigen for 6 days. Cultures were pulsed with 0·25 μCi of tritiated thymidine (TRA 61, Radiochemical Centre, Amersham, UK 5 Ci/mmol) for 8 h, then harvested onto glass fibre discs for liquid scintillation counting. Wells without antigen and wells with VZV control antigen were also run. Results represent experimental minus background counts (Δ ct/min) for triplicate wells.

Preparation of target cells. Target cells were prepared from marrow fibroblasts cultured from routine bone marrow aspirates. Details of the culture and infection of these cells are given elsewhere (Bowden *et al.*, 1984). Briefly, heparinized bone marrow aspirates were cultured in Dulbecco's MEM with 15% FCS. Non-adherent cells were washed off after 5–7 days and the adherent fibroblasts were continued in culture for 2–3 weeks until a confluent monolayer was formed. The cells could then be trypsinized and subdivided.

Infected targets were prepared from confluent monolayers in 25 cm^2 Costar flasks. In preliminary experiments we used cell free VZV added at an MOI of 0·1 which gave 96–100% CPE after 96 h. Subsequently we used 0·2 ml of cell associated virus prepared as described above to infect target fibroblasts. This latter method gave a more homogenous infection that permitted harvesting of the monolayer after 48 h. Indirect fluorescent staining confirmed greater than 90% infection.

Targets were labelled by trypsinizing the monolayers, washing them and suspending 10^6 cells in

0·3 ml of medium with 10% FCS containing 100 μCi of ^{51}Cr (New England Nuclear, 1 mCi/ml). The cells were incubated at 37° for 40 minutes and washed three times in RPMI 1640 with 20% FCS. Viability was greater than 90% by eosin Y exclusion. The cells were then suspended to 5×10^4/ml.

Chromium release cytotoxicity assay. Fresh effector cells were tested in an 18 h ^{51}Cr release assay by standard methods in a round bottom 96 well microtitre plate (Linbro, Hamden, Connecticut, USA: No. 76-013-05). Five thousand target cells in 0·1 ml were mixed with 0·1 ml of effectors (both in RPMI 1640 with 10% FCS) to give effector:target (ET) ratios at 50:1, 20:1, 10:1, and 2:1. All cultures were run in triplicate at 37°C. After 18 h the plates were centrifuged (at 4°C, 150*g*, 5 min) and 100 μl of supernatant was harvested and counted in a gamma counter.

The specific lysis was calculated from mean values according to the following formula:

$$\% \text{ SL} = \frac{\text{test ct/min} - \text{control ct/min}}{\text{maximum ct/min} - \text{control ct/min}} \times 100.$$

Test ct/min were ^{51} Cr counts released by targets in the presence of effector cells, control ct/min were from targets in absence of effector cells, and maximum ct/min were from frozen–thawed target cells. Uninfected targets were run in parallel with infected targets at identical ET ratios in all experiments. Targets and effectors were used in various HLA matched and unmatched combinations.

Blocking with antibodies. Antibodies tested for blocking the cytotoxic response were anti-Ia, (BRL Molecular Diagnostics No. 9408SA), anti-HLA framework antibody (obtained from Dr John Kearney, Birmingham, Alabama), and Leu 2a and 3a (Becton–Dickinson Lot No. CO704), T4 and T8 (Kung *et al.*, 1979), M1 (Zarling & Kung, 1981) (Ortho monoclonal antibodies), HNK 1 (Abo, Cooper & Balch, 1982) and Varicella zoster immune globulin (Lot M-VZIG-8). Before using, the monoclonal and VZV antibodies were diluted 1:10 and dialysed against HBSS for 8 h. The anti-Ia antibody was added directly to prepared cytotoxicity wells for a final dilution of 1:1,000. Leu 2a (0·02 mg) or Leu 3a were incubated with 3×10^6 effector cells on ice for 30 min and washed twice with HBSS before use. Anti-HLA framework antibody (4 μg) were incubated directly with 6×10^4 chromated target cells at 37°C for 30 min and washed twice with HBSS.

Selective removal of T cell subsets. MNC subpopulations were selectively removed by panning (Mage, McHugh & Rothstein, 1977) using T antibodies and Petri dishes (Falcon 2021) pre-coated with affinity purified goat anti-mouse IgG (Tago 4150). Less than 5% of the cells adhered under the control (no T antibody) conditions. The control panned cells were counted and resuspended to 10^6/ml for use as effector cells. Cells pre-treated with antibody were adjusted to an identical volume so as to retain the original concentration of unadhered cells.

Cold target inhibition studies. Cold target inhibition studies were at a 50:1 ratio essentially as described by Braciale (1977) for influenza virus. The method was modified to allow longer pre-culture periods to infect the cold targets; 2 days for infection by VZV or 10 days for infection of cold targets by the AD 169 strain of CMV.

RESULTS

Contribution of HLA matching to lysis of VZV infected targets

The cytotoxic activities of various combinations of HLA matched and unmatched targets were studied to determine whether the lysis of target cells was HLA restricted. Table 1 summarizes results of autologous, partial HLA matched (defined as a match with 1 HLA A or B locus in common) and total mismatched combinations of effectors and target cells. Maximal cytotoxicity was observed with the autologous combinations, while cytotoxicity in the totally unmatched setting was less than 10% (and near that of control) except at the highest E:T ratio of 50:1. Intermediate killing was seen with the partially HLA matched combinations. Fibroblasts used in the autologous combinations served as partially matched or unmatched targets in combinations with other donors and the degree of lysis corresponded with the level of matching. There was no evidence of different susceptibility to lysis of the different VZV infected fibroblast lines. This data demonstrated that VZV specific target cell lysis was more efficient when at least one class I MHC antigen was shared between the effectors and the targets.

Table 1. Killing of autologous, HLA partially matched and HLA mismatched VZV infected targets*

		% specific lysis at E:T ratios of			
		50:1	20:1	10:1	2:1
Autologous targets	VZV+	34(6)	33(4)	21(2)	9(1)
	VZV−	8(2)	6(1)	3(1)	2(1)
Partial match targets	VZV+	26(3)	20(2)	12(2)	4(1)
	VZV−	4(1)	4(1)	4(1)	4(1)
Mismatch targets	VZV+	18(3)	7(2)	6(1)	4(1)
	VZV−	2(1)	0	0	0

* ^{51}Cr release assay as in Materials and Methods. Partial match defined as an E:T combination with one or two HLA antigens in common. Results are the mean, with standard error in parentheses, of seven autologous combinations eight partial matches and eight mismatched combinations.

Characterization of effector cells

Cytotoxic T lymphocytes (CTL), natural killer (NK) cells and monocytes might each contribute to the lysis of VZV infected fibroblasts. Participation of CTL is suggested by the increased lysis seen where effectors and targets shared one or more HLA antigens. Further evidence to implicate CTL in lysis came from inhibition following the addition of Leu 2a or Leu 3a to the cultures for the 18 h cytotoxicity assay (Table 2). Target cell lysis was also inhibited when the effector population was depleted (by panning) of cells bearing the T8 antigen or when the targets were pre-treated with anti-HLA antibody (Fig. 1). Pre-treatment of the effectors with anti-HLA did not inhibit

Table 2. Inhibition of lysis of VZV infected fibroblasts during culture with blood mononuclear cells*

Antibody	Percentage inhibition
Leu 2a	52±4
Leu 3a	45±5
Leu 2a+Leu 3a	81±4
Anti-Ia	12
Anti-HLA framework	53±4
VZIg	range (0−2−+4)

* Results are the mean of two to four experiments per inhibitor using different donors with HLA matched VZV infected targets at a 20:1 E:T ratio and an 18 h culture period. The source and concentration of inhibitors is described in the methods section. Percentage inhibition is calculated as

$$\frac{\text{control}-\text{test}}{\text{control}} \times 100.$$

and a standard error is shown where the experiment was repeated three or more times. The mean control specific lysis in these experiments at 50:1 ratio was 42% (s.e. = 5), 32% (s.e. = 4) at 20:1, with less than 10% lysis of uninfected targets in all combinations.

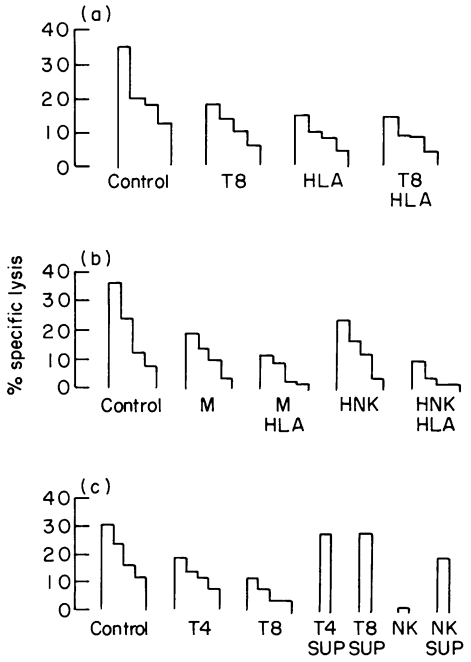

Fig. 1. Lysis of VZV infected fibroblasts by blood MNC sharing two HLA A/B antigens with the targets. Each block shows specific lysis at 50:1, 20:1, 10:1 and 2:1 E:T ratios in an 18 h ^{51}Cr release assay. (a) T8 = effector cells depleted of $T8^+$ cells; HLA = target cells pre-treated with anti-HLA A/B antibody; T8 HLA = both procedures applied. (b) M = effector cells depleted of $M1^+$ cells; HNK = depleted of HNK 1^+ cells. (c) T4 = effector cells depleted of $T4^+$ cells; T8 = depleted of HNK 1^+ cells; SUP = addition of lymphokine containing supernatant; NK = effector cells enriched for NK cells by gradient centrifugation. Mean spontaneous release in these experiments = 26% with < 4% replicate variation.

cytotoxicity (data not shown). This argues against the formation of antigen–antibody complexes and blockade of Fc receptors as the only mechanism for inhibition of lysis. Furthermore, when HLA A2, 3, B7, 60 VZV infected targets were incubated with A2, 3 B7, 12 effectors the addition of an anti HLA A2 antibody (supernatant of clone PA 2.1, ATCC No. HB 117) did not block killing (specific lysis at 20.1 E:T ratio 22% without antibody, 21% with antibody).

Some lysis of target cells persisted at all E:T ratios whether T8 depletion, anti-HLA or both were used. NK cells or monocytes were candidate effectors for this persisting killing. Evidence to support this came from the inhibition of lysis when the effectors were depleted of a subset of NK cells with HNK 1 or depleted of monocytes and NK cells with M1 (Fig. 1). Maximal inhibition of lysis occurred when the effectors were depleted with HNK 1 or M1 and the targets were pretreated with anti-HLA antibody: this combination would be expected to inhibit both CTL and NK mediated cytotoxicity. The specific lysis at 50:1 and 20:1 E:T ratios in this and 2 further experiments gave six points for comparison of inhibition by M *vs* M + HLA and HNK *vs* HNK + HLA. In each case the addition of anti-HLA significantly increased the inhibition of cytotoxicity ($P \leqslant 0{\cdot}05$ for a two tailed rank sum test). Addition of VZIG to the cultures did not impair lysis.

Specificity of target cell lysis

The results in Table 1 suggest that the effector cells had specificity for both the target cell HLA and a virus product. When, in preliminary studies, cold target inhibition was attempted to confirm this possibility, there was no clear superiority of HLA matched over HLA unmatched VZV infected competitors. Lysis of the targets by NK cells or monocytes in the fresh blood MNC effector population was a possible confusing factor (see Zarling & Kung, 1980) so inhibition was re-studied

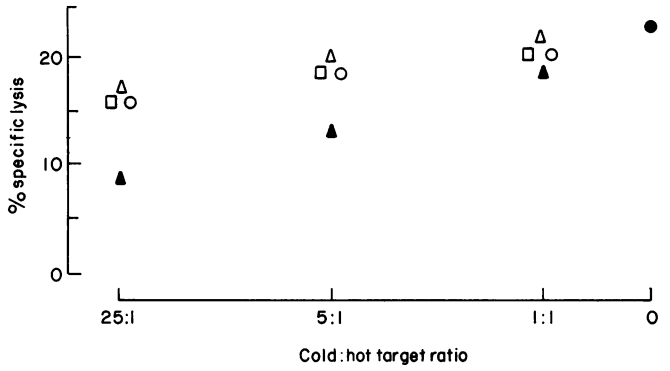

Fig. 2. Cold target inhibition of lysis of VZV infected fibroblasts by blood MNC sharing two HLA A/B antigens with the targets. The cold targets were: ▲ = matched, VZV infected; △ = matched, CMV infected; □ = unmatched, VZV infected; ○ = unmatched, CMV infected. Data shown is at a single E:T ratio of 50:1 using effectors previously depleted of $M1^+$ cells by panning. Specific lysis in the absence of cold targets was 25% (●).

using effectors depleted of M1 positive cells. In the representative experiment shown (Fig. 2) maximal suppression of lysis occurred when the cold targets were both HLA matched and VZV infected. The cold targets in this experiment were infected with CMV to confirm that the effector cells were also specific for the appropriate virus.

Soluble factors in the amplification of lysis

Lysis of VZV infected fibroblasts was inhibited when Leu 3a was added to the cultures (Table 2) or $T4^+$ cells were removed by panning (Fig. 1). The inhibition which followed $T4^+$ or $T8^+$ cell depletion was reversed by addition of a PHA-induced (but mitogen free) lymphokine containing supernatant (Kurnick *et al.*, 1979) to the cultures. These results suggested that soluble factors enhanced target cell lysis, as might occur if CTL were helped or NK cells stimulated. The latter possibility is supported by the finding that target cell lysis by a Percoll gradient NK enriched population was increased in the presence of soluble T cell factors (Fig. 1).

Cytotoxicity by pre-stimulated effector cells

Previous studies indicate that pre-stimulation of effector cells enhanced HLA restricted specific cytotoxicity for herpes simplex or Epstein–Barr virus infected targets. In our initial experiments we therefore used pre-stimulated blood MNC as effector cells in a 4 h cytotoxicity assay but we found that the pre-stimulated cells lysed targets without specificity for HLA or virus infection (Table 3). Tetanus toxoid pre-stimulated MNC behaved in a similar non-specific fashion. Because the cytotoxicity by prestimulated MNC was non-specific we used only fresh MNC for our patient studies.

Patient studies

We studied patients with 3 months to 2 years of remission from acute leukemia: most were receiving only 6-MP. Their VZV antibody titres, lymphocyte proliferation to VZV antigen and cytotoxicity by ^{51}Cr release is shown in Table 4. Adult controls are shown as control cytotoxicity data in children are not available. Patient Nos 1–4 had no history of chickenpox infection and no VZV antibody by FAMA test; patients 5–13 were VZV antibody positive. Of these, patients 5–7 had reduced lymphocyte proliferation as compared with our age matched controls while patients' 8–13 lymphocytes proliferated normally. One of the patients (No. 5) with low VZV specific T cell proliferation had cytotoxicity within the range found in healthy controls. The remaining two patients had low proliferation and four of the patients with normal proliferation had reduced cytotoxicity. Patient 13 who had normal proliferation and high levels of cytotoxicity had been off all anti-leukaemic treatment for 6 months. None of the patients had circulating blasts at the time of

Table 3. Target cell lysis by pre-cultured MNC*

Stimulus	Targets	% lysis at E:T ratios of: 50:1	20:1	10:1	2:1
VZV	VZV+	52	40	25	9
	VZV−	38	24	16	7
TET TOX	VZV+	27	25	11	0
	VZV−	26	28	15	5
0	VZV+	8	4	5	4
	VZV−	6	6	5	5

Results are the mean of three experiments using MNC pre-cultured for 6 days and incubated with HLA unmatched fibroblast targets for 4 h.

Table 4. Lymphocyte proliferation versus cytotoxicity to VZV in patients on maintenance therapy for acute leukaemia

Patient	VZV FAMA titre*	VZV response (ct/min)†	Lymphocyte count‡	Cytotoxicity (E:T ratio)§ 50:1	20:1	10:1	2:1
(1)	−	0	2,981	21	18	14	6
(2)	−	0	1,029	21	15	6	1
(3)	−	0	1,431	9	16	11	10
(4)	−	0	3,912	3	4	0	0
(5)	+	3,275	1,232	32	24	16	8
(6)	+	2,888	480	8	6	5	3
(7)	+	1,693	400	9	8	7	7
(8)	+	7,182	1,062	6	11	5	0
(9)	+	7,729	1,066	13	9	10	1
(10)	+	6,824	1,322	22	20	13	3
(11)	+	5,881	858	10	7	1	2
(12)	+	5279	2,009	17	6	6	4
(13)	+	13,479	2,021	50	46	31	6
Adult controls	+	8,817		34	33	21	9
s.e. of mean		220		6	4	2	1

* Defined as titre $\geqslant$ 1:2.

† Normal values were obtained from studying 12 age matched normal healthy children whose ct/min ranged from 4300 to 13,500 with a mean value of 9,800. Decreased ct/min defined as 4,800 = mean − 2s.d.

‡ Blood lymphocyte count per mm^3.

§ These values represent %SL are derived in the same way as for Table 1. Values for uninfected targets (data not shown) was less than 10% for all patients at all ET ratios.

testing. Our results are consistent with the possibility that the immunosuppressive effects of anti-leukaemic treatment interfere with VZV specific cytotoxicity.

DISCUSSION

Marrow derived fibroblasts have clear advantages as target cells for cytotoxicity experiments. They bear class I MHC (HLA, A,B) but not II (Ia) antigens (Wernet, 1976) and are readily infected by viruses.

We found that VZV infected fibroblasts were lysed by at least two mechanisms. Lysis by freshly drawn blood mononuclear cells was best demonstrated in an 18 h culture period and was optimal when the effectors and target cells shared one or more class I HLA antigens. The requirement for HLA A or B sharing and inhibition by anti-HLA antibodies are characteristics of lysis mediated by cytotoxic T lymphocytes (CTL). Leu 2a antibodies bind to class I MHC restricted T cells (Ledbetter *et al.*, 1981) so the inhibition of cytotoxicity we obtained with this antibody was expected. In common with studies in which alloantigen was used as targets we did not succeed in blocking all cytotoxicity with anti-subset antibodies (Malissen *et al.*, 1982). Inhibition by Leu 3a or by panning with T4 was unexpected as this antibody binds to a T cell subset which is thought to be restricted to I region associated responses (Meuer *et al.*, 1983). Since the target cells were Ia antigen negative it seems unlikely that the Leu $3a^+$ $T4^+$ cells would kill them directly, unless they are first induced to become Ia^+. The contribution of Leu $3a^+/T4^+$ cells more likely involves the production of soluble mediators including lymphokines (Henney *et al.*, 1982) and interferon (Herberman, Ortaldo & Bernard, 1979). A requirement for helper factors for the development of mouse (Raulet & Bevan, 1982), rat (Bellgrau, 1983) and human (Friedman & Thompson, 1983) CTL during several days' *in vitro* culture is already established, but we cultured for only 18 h. This would be insufficient for the proliferation of CTL precursors though it would suffice for the production of mediators such as interleukin-2.

The lysis of herpes virus infected fibroblasts by NK cells (Bishop, Glorioso & Schwartz, 1983) could suggest a role for natural killing in VZV specific cytotoxicity, possibly enhanced by interferon (Herberman *et al.*, 1979). Our observation that effector cell depletion with anti-NK cell antibodies (HNK 1 and M1) partially inhibited killing is consistent with this view. Even effector cell populations depleted of T cells by panning for T4 and T8 positive cells, or Percoll gradient enriched populations of NK cells, could be induced to kill when lymphokine containing supernatants were added to the cultures. Under these circumstances one would expect that any stimulus which activated T cells might result in increased target lysis by NK cells. The HLA unrestricted lysis we found using prestimulated MNC probably illustrates this effect.

The observation that preculture of MHC or recent infection was not required in order to obtain specific lysis of VZV targets suggests that the cytotoxic precursor cell frequency in the blood of immune individuals was high. Although we did not undertake formal limiting dilution of the effector cells to establish a precursor cell frequency it was our impression that ^{51}Cr release from infected targets became more erratic as the ET ratio was reduced to 10:1 (5×10^4 effectors per well). Occurrence of lysis at this effector cell number suggests that at least 1 in 5×10^4 blood MNC from VZV immune individuals can kill a VZV bearing target. This frequency is close to the $1:10^4$ cells which we have previously determined to respond to VZV antigens by proliferation (Giller *et al.* 1983). A likely explanation for the high responder cell frequency for VZV is that the virus usually persists in the body after clinical chickenpox infection. Reactivation of VZV (usually in the form of Herpes zoster) is common in immunosuppressed patients and in previous studies there were no clear correlations between T cell proliferation response to VZV and reactivation. Our present results provide a possible explanation for this lack of correlation: that specific cytotoxicity may be inhibited independently of proliferation by helper T cells.

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