

## Cellular interactions in the lysis of varicella-zoster virus infected human fibroblasts

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### SUMMARY

The *in vitro* lysis of varicella-zoster virus (VZV) infected human fibroblasts by blood mononuclear cells (MNC) is inhibited by cyclosporin A, whether or not the effector and target cells share HLA A or B antigens. Interleukin 2 (IL-2) reversed the inhibition by cyclosporin A (CyA) and also induced a further increase in target cell lysis by MNC in the absence of CyA. MNC depleted of OKM-1<sup>+</sup> or Leu-11<sup>+</sup> cells showed reduced lysis of VZV infected fibroblasts and this reduction was not overcome by adding IL-2. Depletion of monocytes from the MNC effectors reduced target cell lysis and this effect was reversed by adding Interleukin 1 (IL-1). The results indicate that NK cells contribute to the lysis of VZV infected cells and suggest that IL-2 release by T cells, as a result of HLA matching or antigen representation, may amplify this mechanism.

**Keywords** NK cells cyclosporin A herpesvirus varicella zoster-virus

### INTRODUCTION

Herpesvirus infected cells express surface antigens which are recognized by natural killer (NK) cells (Bishop *et al.*, 1983; Fitzgerald *et al.*, 1983) and, in conjunction with class I histocompatibility antigens, by cytotoxic T lymphocytes (CTL) (Yasukawa *et al.*, 1983). When blood mononuclear cells (MNC) are used to lyse infected target cells *in vitro* it is difficult to distinguish between these two effector populations. One approach is to compare the lysis of HLA matched (recognized by CTL + NK cells) and unmatched (recognized by NK cells) targets (Quinnan *et al.*, 1981). We previously found that the lysis of varicella zoster virus (VZV) infected fibroblasts was greater when MNC and targets shared two or more HLA antigens (Bowden *et al.*, 1985) but that equivalent inhibition of lysis was achieved with antibody depletion of CTL or NK cells. Since the inhibition was increased when both antibodies were used compared with either singly we suggested that both cell types contributed to *in vitro* target cell lysis, acting, as shown in other systems, either direction or through lymphokine release (Yasukawa & Zarling, 1984; Brooks, 1983). Lymphokines are known to amplify target cell lysis by NK cells (Herberman, Orotaldo & Barnard, 1979) and by a population of less well defined 'anomalous' killers (Grim *et al.*, 1982). In the studies reported here we use Cyclosporin A, recombinant IL-2, depletion of monocytes and anti NK antibody (Leu-11 and OKM-1) to provide support for the role of lymphokine amplified NK cells in the lysis of VZV-infected targets *in vitro*.

## MATERIALS AND METHODS

*Target and effector cells.* Target cells were bone marrow fibroblasts grown to confluence and infected with the CP5,262 strain of VZV as previously described (Bowden *et al.*, 1984). The effector cells were MNC separated from the blood of healthy VZV-immune donors on Ficoll-Hypaque. All cell washes were in HEPES buffered Hanks balanced salt solution (HBSS) and cultures were in RPMI 1640 + 10% fetal calf serum and 40 µg/ml gentamicin. Specific NK subsets were depleted from the effector population by panning (Mage, McHugh & Rothstein, 1977). MNC were incubated with Leu-11 (Becton Dickinson, Erembodegem, Belgium) at 5 µl per 10<sup>6</sup> cells, HNK-1 (ATCC), 100 µl supernatant per 10<sup>7</sup> cells, OKM-1 (Ortho Reagents, Raritan, NJ, USA) at 20 µl per 10<sup>7</sup> cells or HBSS on ice for 30 min. The cells were washed three times and separated on petri dishes (Falcon 1007) coated with goat antimouse antibody (Tago No. 4140 anti IgG for OKM-1; No. 4152 anti IgM for Leu-11 and HNK-1). After 3 h the non-adherent cells were recovered by pipetting, counted and adjusted to 2.5 × 10<sup>6</sup> cells/ml. Blood MNC were HLA typed by cytotoxicity using NIH basic or Terasaki trays.

*Cytotoxicity assays.* Fibroblasts were detached with trypsin and labelled with <sup>51</sup>Cr as previously described (Bowden *et al.*, 1984). Cytotoxicity tests were performed in Flow microtitre trays (Linbro 76-012-05) with 0.1 ml of MNC at varying concentrations and 0.1 ml of fibroblasts (2.5 × 10<sup>4</sup> cells/ml) each in RPMI 1640 + 10% fetal calf serum per well. MNC were suspended at 2.5 × 10<sup>6</sup> cells/ml, to give a maximum effector/target (E/T) ratio of 100:1. Cultures were incubated in 5% CO<sub>2</sub> in air at 37°C for 18 h and then centrifuged at 200 g for 10 min. 0.1 ml of the supernatant was removed for gamma counting. Percentage specific lysis was calculated as:

$$\text{Percentage specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}} \times 100$$

*Monocyte depletion and lymphokines.* MNC were depleted of monocytes by passage of 10<sup>7</sup> cells over a 3 ml column of Sephadex G-10 (Pharmacia, Piscataway, NJ, USA) packed in a syringe as described by Mishell, Mishell & Shigii, 1980. The non-adherent cells were eluted in HBSS and adjusted to 2.5 × 10<sup>6</sup> cells/ml: <2% of these cells adhered to plastic or had monocyte morphology. IL-1 (Genzyme, Boston, USA, Cat. No. GUPI 1) was used at 1 u/ml and IL-2 (Amgen, Thousand Oaks, CA, USA, Cat. No. 01010) at 1 u/ml. Cyclosporin A was dissolved in DMSO at 1 mg/ml and diluted in RPMI 1640.

## RESULTS

*Cyclosporin A inhibits lysis of VZV infected fibroblasts*

In preliminary experiments the effect of 0.5, 1 and 2 µg/ml of cyclosporin A (CyA) on the lysis of VZV infected bone marrow fibroblasts by blood MNC was determined. The results (Table 1) indicate that the inhibition of target cell lysis is dose related. We selected 2 µg/ml for further experiments; this concentration is similar to that employed by others (Hess & Tutschka 1980).

In experiments where targets matched for 2 HLA A or B antigens with the effector MNC were compared with unmatched effector: target combinations, the lysis of each was inhibited by CyA (Table 2). Cyclosporin A is known to inhibit lymphokine production by T cells (Andrus & Lafferty, 1982) so it was possible that this action contributed to the inhibition of lysis observed. We therefore added 1 u/ml of recombinant IL-2 to the cultures and found (Table 2 lines 3 & 6) that the level of target cell lysis was increased to, or above, the previous levels. The occurrence of this increase, whether or not effectors and targets were HLA matched, suggested that NK cells were involved in lysing the target cells. We tested this possibility by depleting the effectors, by panning, of different NK subsets.

*Leu 11 cells are required for IL-2 mediated amplification of lysis*

MNC in these experiments were either unmanipulated (control), panned without monoclonal antibodies (panned control) or depleted of NK cell subsets by adherence to goat anti-mouse coated

**Table 1.** Inhibition of lysis of VZV-infected fibroblasts by cyclosporin A\*

Cyclosporin A concentration	Percentage specific lysis at E/T ratios of:			
	100:1	50:1	25:1	13:1
0	64	52	48	20
0.5	52	52	36	19
1	48	30	30	11
2	35	20	14	11

\* Means of two experiments with effectors and targets sharing three HLA antigens. Lysis of uninfected fibroblasts was 10%. Cyclosporin A was added at the start of culture, concentrations shown are  $\mu\text{g/ml}$ .

**Table 2.** IL-2 reverses the inhibition of lysis of VZV targets caused by CyA\*

Targets	Culture	Percentage specific lysis at different E/T ratios			
		100:1	50:1	25:1	13:1
Matched	Control	41	28	19	12
	CyA	20	13	11	6
	CyA + IL-2	42	31	16	9
Unmatched	Control	30	20	11	7
	CyA	15	18	11	6
	CyA + IL-2	40	30	16	10

\* Means of four experiments. Standard error ranges were 4-6 at 100:1; 2-5 at 50:1; 1-3 at 25:1 and 2-3 at 13:1 ratios. Lysis of uninfected matched or unmatched targets was < 10%. Cyclosporin A (CyA) at 2  $\mu\text{g/ml}$ , IL-2 at 1 u/ml.

petri dishes following preincubation with HNK-1, OKM-1 and Leu-11 monoclonal antibodies. The panning step depleted 70-90% of the cells which were coated with the monoclonal antibodies as judged by second antibody staining with fluorescein conjugated goat-anti-mouse Ig and flow cytometry analysis.

The inhibition of target cell lysis by panning alone was variable and ranged between 10 and 40% (compare the panned control lines 1 & 2 of Table 3 with Tables 1 & 2). Preincubation of the effectors with HNK-1 before panning increased the inhibition of lysis, though the effect was greater at the 50:1 than 100:1 E/T ratio. Target cell lysis was more markedly inhibited by depleting the effectors of Leu-11<sup>+</sup> or OKM-1<sup>+</sup> cells. Addition of IL-2 to the control panned or HNK-1 depleted cells resulted in increased target cell lysis. This effect of IL-2 was lost when the effector cells were predepleted of OKM-1<sup>+</sup> or Leu-11<sup>+</sup> cells. These results indicated that the cells whose effector function is amplified by IL-2 bear the Leu-11 and OKM-1 antigens, but apparently not the HNK-1 antigen. To investigate the modest reduction in target cell lysis which resulted from control panning we Giemsa-stained and examined the cells which had adhered: over 90% were monocytes.

**Table 3.** IL-2 mediated amplification of lysis requires OKM-1<sup>+</sup> and Leu-11<sup>+</sup> cells\*

E/T ratio		Percentage specific lysis							
		Without added IL-2				With 10 u/ml IL-2			
		100	50	25	13	100	50	25	13
Panned control	M	30	20	16	8	40	28	25	13
Panned control	U	19	7	5	3	31	18	14	5
HNK-1 <sup>-</sup>	M	28	12	2	2	46	23	12	6
HNK-1 <sup>-</sup>	U	15	13	9	5	41	21	5	1
OKM-1 <sup>-</sup>	M	20	15	12	6	19	12	6	3
OKM-2 <sup>-</sup>	U	7	0	2	2	10	4	2	2
Leu-11 <sup>-</sup>	M	20	14	9	7	15	14	13	9
Leu-11 <sup>-</sup>	U	18	11	6	4	11	6	2	2

\* Results are percentage specific lysis from VZV-infected matched (M) or unmatched (U) fibroblasts after culture with MNC either control-panned (panned) or depleted by panning of HNK-1<sup>+</sup>, OKM-1<sup>+</sup> or Leu-11<sup>+</sup> cells. Results are the means of four experiments: standard errors are omitted for clarity but were  $\pm 4-6$  at 100:1 ratio,  $\pm 4-5$  at 50:1 and  $\pm 2-3$  at 25:1 and 13:1.

**Table 4.** Effect of monocyte depletion on VZV-target cell lysis\*

Targets	Effector cells	Percentage specific lysis at E/T ratios of:			
		100:1	50:1	25:1	13:1
Matched	MNC	47	22	19	20
	G10 depleted	28	18	15	21
	G10 depleted + 1L-1	42	31	16	4
Unmatched	MNC	30	18	2	0
	G10 depleted	5	1	1	1
	G10 depleted + 1L-1	3	1	1	1

\* IL-1 added at 1 u/ml for the duration of the culture. Results are the means of two experiments.

#### *Effect of monocyte depletion on lysis of target cells*

Monocytes could contribute to target cell lysis either directly or through the production of IL-1. To distinguish between these possibilities we depleted blood MNC of monocytes by passage over a G-10 column. Target cell lysis by these cells was diminished compared with undepleted controls (Table 4). When IL-1 was added back to the cultures the lysis of target cells was increased in the matched but not unmatched combinations (Table 4, line 3).

## DISCUSSION

These experiments were undertaken to define a role for NK cells in the *in vitro* lysis of VZV infected fibroblasts by blood mononuclear cells. Our previous results showed that lysis of HLA matched VZV infected fibroblast targets was partially inhibited by antibodies to T4<sup>+</sup> as well as T8<sup>+</sup> lymphocytes, as well as by OKM-1. VZV-infected fibroblasts are not Ia<sup>+</sup> by immunofluorescence (unpublished observation), so it seemed unlikely that they would be lysed by class II restricted T4<sup>+</sup> lymphocytes. An alternative explanation was that T cells responding to VZV in the presence of IL-1 might release lymphokines which would stimulate lysis by NK cells. Direct recognition of VZV by T8<sup>+</sup> cells would be possible if the effectors and targets were matched for the appropriate class I histocompatibility (MHC) antigens. VZV recognition by class II restricted cells would most probably be through antigen represented on monocytes. Studies currently in progress in our laboratory show that monocyte reprocessing of VZV antigen does indeed occur *in vitro* (Pontesilli *et al.*, 1985) so both T4<sup>+</sup> and T8<sup>+</sup> cells are possible sources of lymphokine in our system. The increased lysis of HLA A or B locus matched over mismatched targets in our system argues for direct antigen recognition by T8<sup>+</sup> cells. Direct target cell lysis by class I restricted T cells also appears possible in that the preferential lysis of matched over unmatched targets persisted even when Leu-11<sup>+</sup> or OKM-1<sup>+</sup> cells had been removed. This view is consistent with our previous report that OKM-1 and anti-HLA framework antibodies inhibited the lysis of VZV infected fibroblasts. Evidence for T4<sup>+</sup> cells as sources of lymphokine for *in vitro* cytotoxicity is less direct and derives from our previous report of inhibition by T4<sup>+</sup> cell depletion (Bowden *et al.*, 1985) and from inhibition by monocyte depletion in our present study. The depletion of monocytes would be expected to remove both antigen processing and IL-1 making cells from the cultures. Adding back IL-1 would not restore antigen processing cells to the culture and so would not be expected to restore IL-2 production and amplified NK cytotoxicity in HLA unmatched combinations. The observed ability of IL-1 to restore cytotoxicity in matched combinations is most likely due to restoring the ability of class I restricted T cells to make IL-2 following antigen recognition. A requirement for IL-1 for the production of IL-2 by cytotoxic T cells has previously been reported (Talmage *et al.*, 1977).

A role for lymphokines in amplifying the lysis of VZV infected fibroblasts is also supported in our experiments by the inhibitory effect of CyA, a fungal product known to inhibit lymphokine release (Andrus & Lafferty, 1982). Additional actions of CyA, such as direct effect on NK cells, have been proposed (Introna *et al.*, 1981) but these appear to have played little if any role in our experiments since the inhibition was entirely reversed by adding back recombinant IL-2.

The panning experiments indicate that Leu-11<sup>+</sup> and OKM-1<sup>+</sup> cells are required for target cell lysis to be increased by added IL-2. Natural killer cells of the Leu-11<sup>+</sup> phenotype were previously shown to lyse fibroblasts infected with herpes simplex virus (Fitzgerald *et al.*, 1983). Our data indicates that Leu-11<sup>+</sup> cells also lyse fibroblasts infected with VZV, another herpes group virus. We conclude that, in the heterogeneous population of cells which make up the blood mononuclear cell fraction, it is difficult to distinguish between lysis directly caused by CTL and that which may be caused by NK cells amplified through IL-2 released by CTL.

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## REFERENCES

- ANDRUS, L. & LAFFERTY, K.J. (1982) Inhibition of T cell activity by cyclosporin A. *Scand. J. Immunol.* **15**, 449.
- BISHOP, G.A., GLORIOSO, J.C. & SCHWARTZ, S.A. (1983) Relationship between expression of herpes simplex virus glycoproteins and susceptibility of target cells to human natural killer activity. *J. exp. Med.* **157**, 1544.
- BOWDEN, R.A., LEVIN, M.J., GILLER, R.H. TUBERGEN, D.G. & HAYWARD, A.R. (1985) Lysis of varicella zoster virus infected cells by lymphocytes from normal humans and immunosuppressed

- pediatric leukemic patients. *Clin. exp. Immunol.* **60**, 387.
- BOWDEN, R.A., MCGAVREN, L., HAYWARD, A.R. & LEVIN, M.J. (1984) Use of bone marrow fibroblasts to prepare targets for an HLA restricted cytotoxicity assay system. *J. clin. Microbiol.* **20**, 696.
- BROOKS, C.G. (1983) Reversible induction of natural killer cell activity in cloned murine cytotoxic T lymphocytes. *Nature*, **305**, 155.
- FITZGERALD P.A., EVANS, R., KIRKPATRICK, D. & LOPEZ, C. (1983) Heterogeneity of human NK cells: comparison of effectors that lyse HSV-1 infected fibroblasts and K562 erythroleukemia targets. *J. Immunol.* **130**, 1663.
- GRIMM, E.A., MAZUMDER, A., ZANG, H.Z. & ROSENBERG, S.A. (1982) Lymphokine-activated killer cell phenomenon. Lysis of natural killer resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes. *J. exp. Med.* **155**, 1823.
- HERBERMAN, R.B., OROTALDO, J.R., & BARNARD, G. (1979) Augmentation by interferon on human natural and antibody dependent cell mediated cytotoxicity. *Nature*, **277**, 221.
- HESS, A.D. & TUTSCHKA, P.J. (1980) Effect of cyclosporin A on human lymphocyte responses *in vitro*. *J. Immunol.* **124**, 2601.
- INTRONA, M., ALLAVENA, P., SPREAFICE, F. & MANTOVANI, A. (1981) Inhibition of human natural killer activity by cyclosporin A. *Transplantation*, **31**, 113.
- MAGE, M.G., MCHUGH, L.L. & ROTHSTEIN, T.L. (1977) Mouse lymphocytes with and without surface immunoglobulin: preparative scale separation in polystyrene tissue culture dishes coated with specifically purified anti-immunoglobulin. *J. Immunol. Meth.* **15**, 40.
- MISHELL, B.B., MISHELL R.I. & SHIGII, J.M. (1980) Adherence. In: *Selected Methods in Cellular Immunology*. (ed. by B.B. Mishell & S.M. Shigii) p. 175, Freeman Press, San Francisco, USA.
- PONTESILLI, O., LASZLO, M., LEVIN, M. & HAYWARD, A. (1985) *In vitro* processing of membrane bound VZV antigens for presentation to T cells. *Fed. Proc.* **44**, 183 (Abstract).
- QUINNAN, G.V., KIRMANI, N., ESBER, E., SARAL, R., MANISCHEWITZ, J.F., ROGERS, J.L., ROOK, A.H., SANTOS, G.W. & BURNS, W.H. (1981) HLA-restricted cytotoxic T lymphocyte and nonthymic cytotoxic lymphocyte responses to cytomegalovirus infection of bone marrow transplant recipients. *J. Immunol.* **126**, 2036.
- TALMAGE, D.W., WOOLNOUGH, J.A., HEMMINGSEN, H., LOPEZ, L. & LAFFERTY, K.J. (1977) Activation of cytotoxic T cells by nonstimulating tumor cells and spleen cell factors. *Proc. natn. Acad. Sci. USA.* **74**, 4610.
- YASUKAWA M., SHIROGUCHI, T. & KABAYASHI, Y. (1983) HLA-restricted T lymphocyte-mediated cytotoxicity against herpes simplex virus-infected cells in humans. *Infect. Immun.* **40**, 190.
- YASUKAWA, M. & ZARLING, J.M. (1984) Human cytotoxic T cell clones directed against herpes simplex virus infected cells. *J. Immunol.* **133**, 422.