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Processing and presentation of cell-associated varicella-zoster virus antigens by human monocytes

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SUMMARY

To determine whether viral antigens associated with infected cells were processed for presentation to T cells, we cultured human blood mononuclear cells (MNC) from varicella-zoster virus (VZV) immune donors with VZV-infected fibroblasts of known HLA type which had been fixed in 0·05% glutaraldehyde. After 7–8 days of culture thymidine uptake by $T4^+$ cells exceeded that of $T8^+$ cells. Stimulated cells were depleted of adherent cells and restimulated with VZV-infected fibroblasts from donors matched or unmatched with the responder for HLA type in the presence or absence of fresh adherent cells. Proliferation of the VZV-specific blasts required the presence of adherent cells matched with the responder lymphocytes for HLA-DR; conversely, the VZV specific response was not restricted by the MHC of the fibroblasts used in the restimulation assay. Preincubation of the adherent cells with chloroquine inhibited the proliferative response in a dose-dependent manner. These results suggest that VZV antigens on infected cells may be processed by monocytes for presentation to T cells.

Keywords varicella-zoster virus antigen presenting cells

INTRODUCTION

The immune response to varicella-zoster virus (VZV) in man has been studied in healthy controls (Jordan & Merigan, 1974; Zaia & Oxman, 1977; Kumagai *et al.*, 1980), and immunocompromised hosts such as leukaemia and lymphoma patients (Patel *et al.*, 1979). Subjects immune to VZV make specific antibodies (Ab) both *in vivo* (Zaia & Oxman, 1977) and *in vitro* (Souhami, Babbage & Callard, 1981), and lyse VZV-infected targets by antibody-dependent cell-mediated cytotoxicity (Babbage, Sigfusson & Souhami, 1984), natural killer (NK) cells and cytotoxic T lymphocytes (CTL) (Bowden *et al.*, 1985; Hayward *et al.*, 1986a, b). Participation of helper T cells (Th) is probably required for the synthesis of Ab (Lane *et al.*, 1981) and the augmentation of NK activity by interleukin 2 (Henney *et al.*, 1982) and interferon (Herberman, Ortaldo & Barnard, 1979). Activation *in vitro* of Th cells requires the antigen to be processed and presented by an antigen presenting cell (APC), bearing the same class II MHC antigens of the T cell (Unanue, 1984). Lymphocyte responses to VZV have been studied using antigen suspensions prepared from VZV-infected cells (Schmidt *et al.*, 1964); little is known about the lymphocyte response *in vitro* to intact VZV-infected cells. In the present study the proliferative response of mononuclear cells (MNC) from VZV-immune healthy donors to glutaraldehyde fixed VZV-infected fibroblasts (VZV-F) was

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analysed. Proliferation was obtained in response to all VZV-F used, both matched and unmatched with the responder MNC for HLA type, and was predominant in the $T4^+$ lymphocyte subset, known to require the antigen to be presented in association with self class II MHC antigens (Engleman *et al.*, 1981). We then investigated the requirements for APC and whether viral cell-associated antigens would be processed by adherent APC. The VZV-infected stimulator cells were fixed to avoid their lysis and to prevent thymidine uptake by the replicating virus. We show here that human monocytes are able to take up, process and present cell associated VZV antigens to T cells in a restimulation assay.

MATERIALS AND METHODS

Subjects. Peripheral blood was obtained from healthy VZV immune adults. Bone marrow samples came from transplantation donors or healthy volunteers. Informed consent was obtained from all subjects. HLA-A, B and DR type was determined by microcytotoxicity assay using fresh MNC and National Institutes of Health basic trays (Amos, Pool & Grier, 1980) and is shown in Fig. 3.

Preparation of VZV-infected fibroblasts (VZV-F). Human bone marrow fibroblasts were prepared and infected with VZV as previously described (Bowden *et al.*, 1984). The fibroblasts, either infected or uninfected, were recovered by treatment for 3 min with 0·05% trypsin in 0·02% EDTA, followed by addition of 5-fold excess of RPMI-1640 (Flow Labs, McLean, VA) with 10% fetal calf serum (FCS). Trypsinized, infected and uninfected fibroblasts from the same batch were washed twice in Hanks' balanced salt solution (HBSS) and then resuspended in 0·05% glutaraldehyde in phosphate buffered saline (PBS). After 30 s on ice an equal volume of 1% glycyl–glycine (Sigma, St Louis, MO) in HBSS was added to neutralize excess glutaraldehyde and the cells were washed twice in HBSS and counted in a haemocytometer. If not used immediately, fixed fibroblasts were stored frozen at $-70°C$ and washed again after thawing. Expression of viral and class I MHC antigens was evaluated by indirect immunofluorescence and fluorescence activated cell sorter (EPICS 'C', Coulter Electronics, Hyaleah, FL) analysis. VZV-immune human gamma-globulin (VZ-Ig) was used to detect VZV proteins on fixed and unfixed fibroblasts together with fluorescein conjugated goat anti-human IgG (Cappel, Cochranville, PA) as second antibody. Expression of class I MHC antigens was assessed by staining with monoclonal antibody W6/32 (obtained from American Type Culture Collection), known to react with a non-polymorphic determinant on the HLA-A, B, C molecules (Brodsky & Parham, 1982), followed by fluorescein conjugated rabbit anti mouse IgG (Cappel).

Soluble VZV-antigen (VZV-Ag) was prepared by glycine extraction from unfixed infected fibroblasts as described by Zaia, Leary & Levin (1978). The VZV-Ag concentration in these extracts was estimated from the number of cells extracted. Glycine extracts of uninfected fibroblasts were used as control antigen.

Lymphocyte stimulation in vitro. MNC were separated on Ficoll-Hypaque (Böyum, 1968), washed twice in HBSS and resuspended in RPMI-1640 (Flow Labs.) supplemented with 2 mM L-glutamine (Flow Labs.), 40 mg/l gentamicin (Garamycin, Schering, Kenilworth, NJ) and 10% pooled human serum (HS). MNC were cultured at 10^6 cells/ml in 0·2 ml RPMI 1640–10% HS in round-bottomed microtitre wells (Linbro, cat No 76-042-05, McLean, VA) for 6 days with different numbers of VZV-F fibroblasts or equivalent dilutions of VZV-Ag. Control cultures with uninfected fibroblasts or equivalent dilutions of control antigen were run in parallel. The cultures were pulsed with 0·5 μCi ^{3}H-thymidine (40 Ci/mmol, Amersham, TRK.637, Arlington Heights, IL) for the last 16 h and then harvested onto glass fibre filters for liquid scintillation counting.

Generation of VZV specific blasts. MNC were cultured in RPMI-1640–10% HS at 10^6 cells per ml in 17×100 mm culture tubes (Falcon, cat. No 2057, Oxnard, CA) in the presence of 2×10^4/ml VZV-F. This ratio of MNC to fibroblasts had been found optimal in the stimulation assay described above. After 7–8 days' culture in 5% CO_2 humidified atmosphere at 37°C, live cells were recovered by centrifugation on Ficoll-Hypaque and depleted of adherent cells by 1 h incubation in plastic Petri dishes (Falcon, cat No 3002).

Phenotype of proliferating cells. MNC cultured with VZV-F infected or uninfected fibroblasts were pulsed on day 6 with ^{3}H-thymidine (40 Ci/mmol; Amersham, TRK.637, 1·25 μCi per 10^6 cells) for 16 h. Viable cells were then recovered by centrifugation on Ficoll-Hypaque, incubated with OKT4 or OKT8 (Ortho, Raritan, NJ) and then analysed by panning for ^{3}H-thymidine incorporation of the two subsets using polystyrene microwells coated with goat-anti-mouse Ig (Cappel) as described by Suez & Hayward (1985).

Preparation of APC. Peripheral blood MNC from healthy donors were incubated at 2×10^6/ml in 25 cm^2 cell culture flasks (Falcon, cat. No. 3013) in RPMI-1640 supplemented with 1% HS for 1 h. Non-adherent cells were then washed out with HBSS and adherent cells (APC) recovered with a cell scraper (Costar, Cambridge, MA).

Restimulation of VZV specific blasts. Twenty-five thousand Ficoll separated VZV-primed MNC were cultured with 5×10^3 fixed fibroblasts (either infected or uninfected) in the presence or absence of 5×10^3 gamma irradiated (3000 R) APC in round-bottomed microtitre wells (Linbro, cat. No. 76-042-05, McLean, VA) in 0·2 ml RPMI-1640–10% HS. The wells were pulsed after 72 h with 0·5 μCi ^{3}H-thymidine (40 Ci/mmol) and harvested 24 h later.

Chloroquine inhibition of VZV-specific blast restimulation. Two hundred thousand 3000 R gamma-irradiated MNC were incubated in flat-bottomed microtitre wells (Linbro, cat No. 76-032-05) in RPMI-1640–1% HS for 1 h at 37°C, non-adherent cells were washed off and 0·1 ml RPMI-1640–10% HS containing chloroquine (Sigma) at concentrations ranging from 0·12 mM to 4 mM were added for an additional 1·5 h. Some wells containing adherent cells which did not receive chloroquine were fixed in 0·05% glutaraldehyde in PBS for 30 s followed by 1% glycyl–glycine. Adherent cells were then washed and incubated with 25×10^3 VZV-specific blasts and 5×10^3 fibroblasts for 72 h. The cultures were pulsed with 0·5 μCi ^{3}H-thymidine (40 Ci/mmol) for further 24 h, harvested and counted as described above. Percent inhibition was calculated as follows:

$$\% = \frac{(\text{ct/min VZV-F} - \text{ct/min uninfected}) \text{ chloroquine}}{(\text{ct/min VZV-F} - \text{ct/min uninfected})} \times 100$$

A similar restimulation assay was performed to investigate chloroquine inhibition of tetanus toxoid (TT) processing. TT was obtained from Wyeth Labs (Marietta, PA) and used at 2 Lf/ml (Lf, limits of flocculation); TT specific T cell lines and clones, obtained from healthy immune donors according to standard procedures (Schmitt *et al.*, 1982), were used as responder cells at the concentration of 10^4 cells/well. Viability of chloroquine pretreated cells was checked by acridine orange/ethidium bromide staining; the number of adherent cells was calculated by counting four microscope fields of known area in four replicate wells for each chloroquine concentration used.

RESULTS

Expression of class I MHC and VZV antigens on fixed fibroblasts. Rapid fixation in 0·05% glutaraldehyde has been reported to leave intact the immunoreactivity of VZV proteins on infected cells (Zaia & Oxman, 1977) and not to interfere with recognition of processed antigens in association with class II MHC antigens (Shimonkevitz *et al.*, 1983). Human fibroblasts do not express class II MHC antigens unless stimulated by gamma-interferon (Pober *et al.*, 1983); similarly, we did not detect HLA-DR antigens on VZV-infected fibroblasts using immunofluorescence techniques (data not shown). To determine whether fixation affected the binding of antibody to cell surface VZV and class I MHC antigens, fibroblasts were stained with VZ-Ig (1 : 100) and anti-HLA-A, B, C monoclonal antibody W6/32 before and after fixation. As shown in Fig. 1, no changes in number and fluorescence intensity of cells stained with W6/32 were observed. Similarly, VZV antigens were expressed with the same density on more than 50% of VZV-F, regardless of the fixation procedure (56·82% positive unfixed VZV-F, mean fluorescence 125; 56·86% positive fixed VZV-F, mean fluorescence 123). The profile of the fixed VZV-infected cells is shown in Fig. 1. The unfixed cells were identical.

Lymphocyte proliferation in vitro *in response to VZV.* We then compared the MNC proliferative response to intact versus solubilized VZV-F. MNC from three donors were stimulated for 6 days

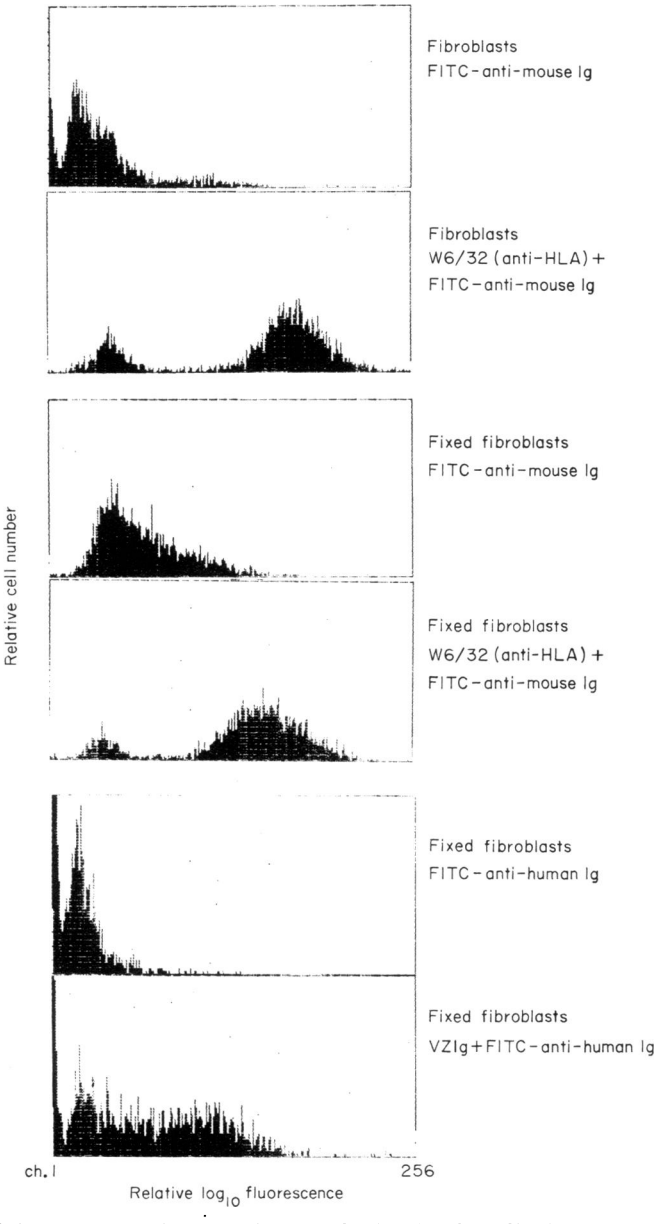


Fig. 1. Expression of class I MHC and VZV antigens on fixed and unfixed fibroblasts. *x* axis: relative $\log_{10}$ fluorescence. Fluorescence intensity is expressed in arbitrary units. The histogram is divided into 256 channels and covers 3 $\log_{10}$ of relative intensity. *y* axis: relative cell number. Vertical bars represent the number of cells in each channel (i.e. with a given fluorescence intensity). The same scale has been adopted in all the histograms shown, each generated by counting 3000 cells.

with intact or solubilized VZV-F or control uninfected fibroblasts, either autologous (two experiments), HLA-A, B identical (two experiments) or HLA-A, B non-identical (five experiments) with the responder cells. As shown in Table 1, VZV-F induced a dose-dependent response both when autologous (or HLA-A, B-identical) and HLA-A, B non-identical with the responder. The magnitude of the response to VZV-Ag was comparable to that obtained with the lowest concentrations of intact fibroblasts (50:1 and 100:1 MNC/fibroblasts ratios) but was not dose-

Table 1. Lymphocyte proliferation *in vitro* in response to autologous or HLA identical and to HLA non identical VZV-F and VZV-Ag*

	MNC/fibroblasts ratio			
	12·5/1	25/1	50/1	100/1
Autologous or HLA-A, B identical fibroblasts (four experiments)				
VZV-F	69·2	51·3	33·1	25·7
(range)	(53·7–89·1)	(40·2–65·5)	(26·3–41·5)	(19·1–34·7)
Uninfected	12·9	9·5	6·6	5·2
(range)	(11·0–15·1)	(8·0–11·4)	(6·1–7·2)	(4·3–6·4)
VZV-Ag†	30·9	28·8	23·9	25·7
(range)	(24·5–38·9)	(21·0–39·5)	(20·4–28·2)	(23·1–28·6)
Control Ag‡	6·7	6·9	6·2	7·6
(range)	(5·5–8·3)	(6·1–7·9)	(5·2–7·4)	(5·6–10·2)
HLA-A,B non identical fibroblasts (five experiments)				
VZV-F	38·0	25·1	22·4	20·2
(range)	(29·9–48·3)	(18·3–34·4)	(17·0–29·4)	15·5–26·2)
Uninfected	11·0	6·2	4·0	3·7
(range)	(8·8–13·7)	(4·8–7·9)	(3·3–4·7)	(2·9–4·8)
VZV-Ag	29·2	28·1	27·5	29·8
(range)	(25·2–33·9)	(23·7–33·3)	(23·5–32·2)	(25·7–34·6)
Control Ag	5·8	5·5	5·6	5·0
(range)	(5·1–6·7)	(4·4–6·9)	(4·7–6·6)	(4·4–5·7)

* Results are expressed as mean (1 s.e. range) of ^{3}H-thymidine uptake (ct/min $\times 10^{-3}$).

† Soluble VZV and control Ag were added to the cultures in concentrations equivalent to the number of fibroblasts used in experiments with intact cells.

‡ Glycine extracts of uninfected fibroblasts.

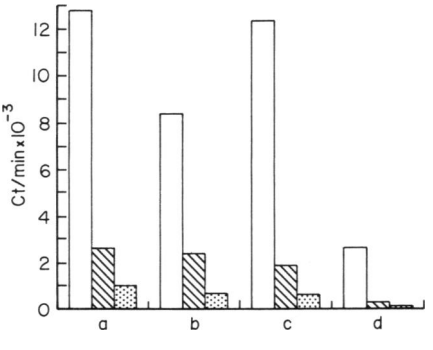
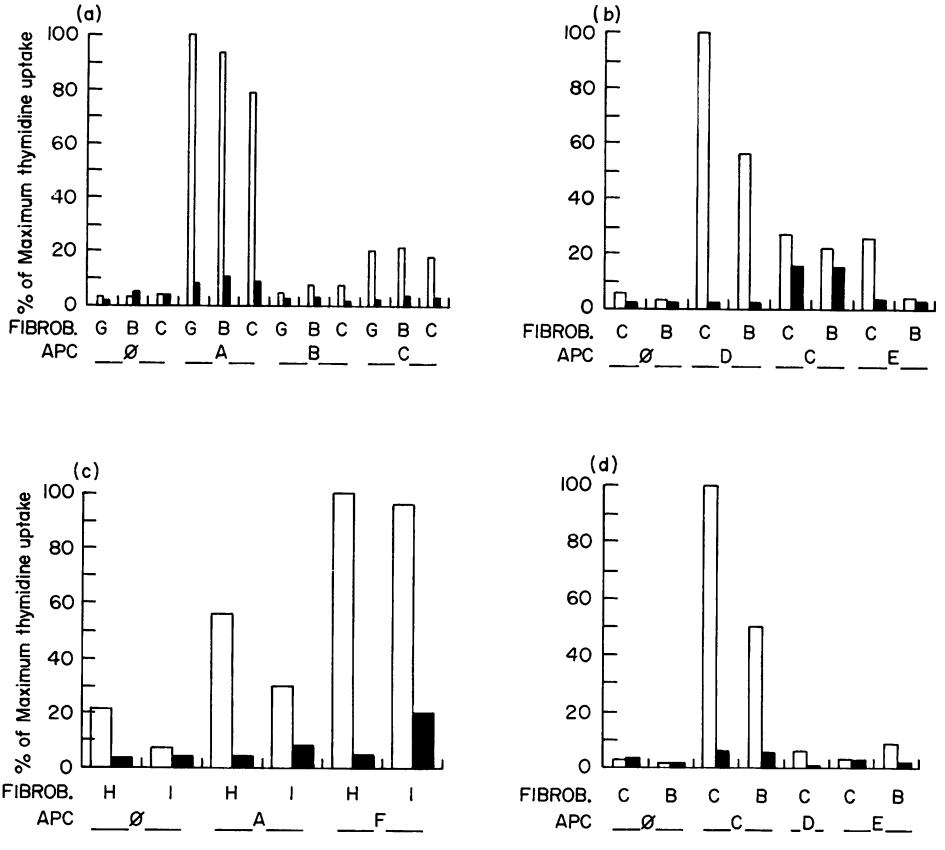


Fig. 2. Thymidine uptake by T4$^+$ and T8$^+$ cells in cultures of MNC with VZV-infected or uninfected fibroblasts. Pulsed MNC were panned on goat anti-mouse Ig coated microwells after incubation with OKT4 (□), OKT8 (▨) or no antibody (▩). The radioactivity that bound to the wells after non-adherent cells were washed out was counted. The fibroblasts used are: (a) autologous VZV-F; (b) HLA-matched VZV-F; (c) HLA-unmatched VZV-F; (d) HLA-unmatched uninfected fibroblasts.



Donor	HLA-A	HLA-B	HLA-DR
A	2, 3	w35, –	5, –
B	2, 3	7, 12	3, 4
C	1, 2	13, –	w6, –
D	2, 3	7, w51	5, –
E	1, w32	17, w52	2, w7
F	w24, w25	18, w35	5, w7
G	2, 3	7, 12	ND
H	1, w31	17, w60	ND
I	2, 9	w21, w35	ND

Fig. 3. Restimulation of VZV-specific blasts with VZV-infected (□) and uninfected (■) fibroblasts and APC from different donors. The combinations of responding MNC and the infected fibroblasts used for generating the specific blasts are: (a) responder A+fibroblasts G; (b) responder D+fibroblasts C; (c) responder A+fibroblasts H; (d) responder C+fibroblasts C. Results are expressed as percent of maximum thymidine uptake obtained in each assay. Table: G, H and I were used as fibroblast donors only. ND, not done.

dependent. The soluble control antigen did not stimulate at any of the dilutions used. The uninfected fibroblasts did induce some proliferation, regardless of their HLA matching with the responder cells. To minimize thymidine uptake in response to the uninfected fibroblasts, further experiments were at an MNC/fibroblast ratio of 50:1.

Table 2. Chloroquine inhibition of VZV- and TT- specific blasts' restimulation

Chloroquine (mM)	APC/well ($\times 10^{-3}$) (mean + s.e.)	APC viability (%)	% inhibition (mean + s.e.)	
			VZV	TT
0	21·4 ± 1·3	97	0	0
0·12	—	—	1·1 ± 6·4	−14·0 ± 0
0·25	—	—	22·1 ± 1·1	—
0·50	18·2 ± 4·5	98	42·2 ± 12·9	40·4 ± 8·6
1·00	20·6 ± 4·5	99	62·5 ± 11·1	61·6 ± 16·6
2·00	27·2 ± 4·4	90	68·0 ± 8·6	63·2 ± 11·8
4·00	25·9 ± 3·1	93	92·3 ± 2·9	—

Phenotype of proliferating cells in culture with VZV-F. To further characterize the response *in vitro* to fixed VZV-infected cells, we determined the phenotype of the cells responsible for thymidine uptake. As shown in Fig. 2, the thymidine uptake by $T4^+$ lymphocytes was greater than that of $T8^+$ lymphocytes, whether the responder MNC and fibroblasts were from the same donor (Fig. 2a, T4/T8 ct/min ratio = 4·98), from HLA matched (Fig. 2b, T4/T8 ct/min ratio = 3·48) or unmatched donors (Fig. 2c, T4/T8 ct/min ratio = 6·45). Thymidine uptake of MNC cultured with HLA unmatched uninfected fibroblasts is shown in Fig. 2d.

Restimulation of VZV specific blasts. The predominant proliferative response of the $T4^+$ subset suggested that monocytes might process the viral antigens and present them in association with class II MHC antigens. We therefore investigated the requirements for restimulation of human MNC primed with VZV-F. VZV specific T cell blasts were restimulated with VZV-F from several donors in the presence of either autologous or heterologous fresh APC. As shown in Fig. 3, autologous APC, or APC matched with the responder for HLA-DR (Fig. 3c), were required to obtain proliferation in response to VZV-F. When DR-unmatched heterologous APC were present, thymidine uptake ranged from 2% to 27% of that obtained in the presence of autologous APC. When autologous or DR-matched APC were present, all the VZV-F tested were able to induce proliferation of VZV-specific blasts, regardless of their HLA type; however, the highest thymidine uptake in each assay was always obtained with the same VZV-F used in the primary culture. No significant restimulation was caused by the control uninfected fibroblasts.

Chloroquine inhibition of VZV specific blasts' restimulation. To investigate whether antigen processing is actually involved in the restimulation of VZV-specific blasts, we performed the restimulation assay described above with APC pulsed with chloroquine, which is known to interfere with the lysosomal proteolysis of macrophages (Seglen, Grinde & Solheim, 1979) and to affect the biosynthesis of Ia molecules (Nowell & Quaranta, 1985). As shown in Table 2, chloroquine treatment of APC before the addition of VZV-F and responder lymphocytes inhibited thymidine uptake in a dose dependent manner. To rule out chloroquine toxicity on monocytes, the number of adherent cells and their viability were checked after 1·5 h incubation with chloroquine and no significant differences in these parameters were observed at the concentrations used. TT specific blasts' restimulation was inhibited by chloroquine in the same extent of the response to VZV, suggesting that both antigens were handled in the same way by the APC. In two experiments where APC were fixed with 0·05% glutaraldehyde before antigen and lymphocyte addition, proliferation was completely abolished (data not shown).

DISCUSSION

Lymphocyte proliferation *in vitro* in response to VZV is usually studied with soluble antigens extracted from VZV-infected cells (Jordan & Merigan, 1974; Kumagai *et al.*, 1980; Chilmonczyk *et al.*, 1985). Cytotoxicity tests, in contrast, use whole cells infected with VZV (Babbage *et al.*, 1984; Bowden *et al.*, 1984; 1985). Our experiments show that VZV-F and soluble VZV-Ag elicit

comparable lymphocyte proliferation. We then determined the phenotype of the cells proliferating in the cultures with VZV-F. Although proliferation of $T8^+$ cells was present, $T4^+$ cells accounted for the majority of thymidine uptake, at least four times greater than that of $T8^+$ cells. Activation of $T4^+$ cells entails the participation of class II bearing APC; therefore we designed a restimulation assay to determine the requirements for sustained proliferation of VZV-specific blasts. Live adherent cells sharing HLA-DR antigens with the responder blasts are required for sustained proliferation regardless of the HLA type of infected cells used both for priming and restimulation. Fixed adherent cells could not restore the response; lysosomal proteolysis seems to be necessary, since chloroquine inhibited antigen presentation. The unavailability of peptide fragments of VZV antigens did not allow us to compare our results with the response to those fragments supposed not to require processing, as shown in other systems (Streicher *et al.*, 1984); however, the similar results obtained with TT strongly suggest that the active participation of APC involves a proteolytic step. The MHC antigens of VZV-F seem not to be the restricting element for the proliferative response to VZV, since both generation of VZV-blasts and their restimulation are achieved with cells from HLA-matched as well as unmatched donors. The finding that the same VZV-F used in the primary culture were responsible for the highest restimulation suggests that the interaction of the viral antigens with the fibroblasts' antigens possibly generates new determinants. However, the fibroblasts' antigens *per se*, including the MHC, are not responsible for this phenomenon since low thymidine uptake was found in control cultures with uninfected fibroblasts, either autologous or differing for one or more HLA-A and B loci with the responder cells. The lack of a proliferative response to alloantigens lead us to conclude that MHC antigens are not processed by the responder haplotype APC in this system. Processing of alloantigens has been reported in mice when live stimulators were used; paraformaldehyde fixation of the stimulator cells abolished the processing of MHC alloantigens by responder haplotype accessory cells leaving their recognition by allospecific CTL intact. The authors concluded that MHC alloantigens need to be shed by the stimulator cells in order to be processed and fixation prevents shedding of surface MHC molecules (Golding & Singer, 1984).

Recent work from our laboratory has shown that IL-2 is required for the full expression of cytotoxicity *in vitro* against infected fibroblasts (Hayward, Herberger & Laszlo, 1986a). Processing of viral antigens by APC and their presentation in association with class II MHC antigens is probably a major mechanism leading to the activation of lymphokine producing T cells.

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