Major Histocompatibility Complex Restriction of T-Cell Responses to Varicella-Zoster Virus in Guinea Pigs

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Varicella-zoster virus (VZV), adapted to grow in guinea pig fibroblasts, was injected subcutaneously into Hartley, strain 2, and strain 13 guinea pigs. Serum immunoglobulin G antibodies were detected 2 weeks later, and T-cell proliferative responses by blood lymphocytes were found 3 weeks after injection. The proliferating cells bound the 155 antibody, which defines a CD4-like subset of guinea pig T lymphocytes. VZV-infected fibroblasts of human, Hartley, and strain 13 origin elicited equivalent amounts of proliferation, which was quantitatively greater than that obtained with an extracted VZV antigen. Uninfected (control) human or guinea pig fibroblasts did not elicit T-cell proliferation. The proliferative response to VZV required the presence of autologous (strain 2 or 13) antigen-presenting cells and was blocked by the addition of an anti-class II major histocompatibility complex antibody. Effector cells obtained from *in vitro* cultures mediated class II-restricted cytotoxicity to L2C cells incubated with VZV. Class I-restricted responses were obtained only by cross-priming strain 2 animals with strain 13 peritoneal exudate cells which had been preincubated with VZV. The data indicate that guinea pigs resemble humans in that class II-restricted T cells with specificity for VZV are more readily cultured from blood than are class I-restricted cells.

Varicella-zoster virus (VZV) causes chickenpox and has the potential to become latent in posterior horn sensory neurones. The reactivation of latent VZV causing herpes zoster (shingles) is a source of serious morbidity due to postherpetic neuralgia. Previous studies have linked the increasing incidence of shingles in the elderly with waning VZV-specific immunity (4, 14), but the point at which VZV-specific immunity is so compromised as to permit VZV reactivation is unknown. VZV-specific cell-mediated immunity can be boosted by immunization with the attenuated Oka strain vaccine (5), but the components of the cell-mediated response which provide protection from VZV are poorly understood. In part this is because there is no generally accepted animal model for the disease. Myers et al. (20) showed that VZV could be adapted to grow in guinea pig cells and that guinea pigs injected with infected cells made VZV-specific antibody responses even though they did not become discernibly ill (21). More recent studies have shown that VZV-inoculated guinea pigs make antibody and T-cell-mediated responses to gpI and to the gene 62 product, p170 (2).

In tissue culture, VZV grows well in a range of cells which express class I major histocompatibility complex (MHC) antigens but are generally negative for class II antigens. Nevertheless, most of the T cells cloned from VZV-stimulated cultures of human mononuclear cells (MNC) have the CD4 antigens which characterize class II-restricted T cells (13) and the cytotoxicity mediated by these cells is class II restricted (11, 15). MHC class I-restricted responses to VZV by human lymphocytes have generally been difficult to detect (16), although recent observations suggest that CD8 cells respond better following depletion of CD4 cells (1). Whether the predominance of class II-restricted T cells in conventional *in vitro* responses to VZV is determined by the

route of antigen presentation during a primary varicella infection or whether it is an artifact of the T-cell culture methods is not known. The issue is important in the context of optimal immunization strategies for the elderly as they become at risk for herpes zoster. Since the route of infection of humans is not open to manipulation, we have used guinea pigs to analyze the MHC restriction of responses to VZV which follow the injection of infected cells into guinea pigs of the strain 2 and 13 inbred lines.

MATERIALS AND METHODS

Guinea pigs and guinea pig cell lines. Strain 2 and 13 guinea pigs were obtained from the Animal Division of the Division of Research Resources of the National Institutes of Health and bred in our animal house. Hartley guinea pigs were obtained locally. Guinea pig fibroblasts (strain 2 and 13 and Hartley) were cultured from the bone marrow of 3- to 4-week-old animals by the method of Bowden et al. (6) and were frozen at passage 5. Guinea pigs were infected by subcutaneous injection of 0.5 ml of a suspension of VZV-infected fibroblasts of the appropriate strain.

Guinea pig macrophages were obtained by intraperitoneal injection of 20 ml of paraffin oil and the harvesting of ascites 5 to 7 days later. Blood was obtained by cardiac puncture of animals anesthetized with Metophane (Pitman Moore, Washington Crossing, N.J.). It was defibrinated and centrifuged to separate cells and serum. The L2C guinea pig lymphoma cell line was generously provided by E. Shevach, National Institutes of Health.

Guinea pig-adapted VZV and antigen. The preparation of guinea pig-adapted VZV is described by Arvin et al. (2). The virus was grown in guinea pig fibroblasts (Hartley, strain 2, and strain 13) to 75% cytopathic effect. The infected monolayers were detached by rolling with glass beads and were then spun down and frozen at -70°C where they were to be used as a source of infectious virus. Monolayers were

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trypsinized and fixed in 0.05% glutaraldehyde for 30 s (terminated by addition of 0.2 M lysine) when they were to be used as a VZV antigen stimulus.

Antibodies to VZV. Guinea pig anti-VZV antibodies were detected by Western blot (immunoblot). Briefly, Hartley strain fibroblasts infected with VZV were lysed in Nonidet buffer and electrophoresed in an 0.8% polyacrylamide gel and transferred onto nitrocellulose in a BioRad apparatus. Strips of antigen were overlaid with the serum to be tested, washed, and then incubated with ^{125}I -labeled protein A (ICN Radiochemicals). The strips were washed and exposed to X-ray film before developing and examination.

Monoclonal antibodies. The 155 antibody is a rat monoclonal antibody identifying a CD4-like epitope on T cells (8). 22C4 is a mouse immunoglobulin G1 (IgG1) which binds to the Ia antigens of strain 2 and strain 13 animals (7). CT6 is a mouse IgG1 which binds to a CD8-like subset (25). For immunofluorescent-staining studies, the mouse antibodies were located with a fluorescein isothiocyanate-conjugated anti-mouse IgG (Boehringer Mannheim, Indianapolis, Ind.) and the rat antibody was located with a mouse anti-rat IgG conjugate (Rockland, Gilbertsville, Pa.).

Cultures. Lymphocytes from immunized and control guinea pigs were obtained either from blood (where serial samples were taken) or from blood and lymph node when the animals were killed. Lymph nodes were teased in Hanks balanced salt solution to obtain a cell suspension. Blood was defibrinated and centrifuged ($600 \times g$, 4 min, 20°C) so that serum could be recovered. The MNC were recovered from a Ficoll-Hypaque gradient (SG 1.088) after 20 min of centrifugation at $770 \times g$ at room temperature. The MNC were washed and adjusted to $10^6/\text{ml}$ in RPMI 1640 medium supplemented with $10 \mu\text{g}/\text{dl}$ of gentamicin and 10% guinea pig serum. The cells were cultured in an upright tube (Falcon 2057; Becton Dickinson) for 4 days and then transferred to a 25-cm^2 flask for 3 more days of culturing, after which 10 U of interleukin-2 [IL-2] per ml was added (ala-IL2, human recombinant; Amgen, Thousand Oaks, Calif.). Cells were removed for testing in cytotoxicity assays at the end of 7 days of culturing with IL-2.

T-cell restimulation assays. T-cell restimulation assays are described by Kurnick et al. (19). Briefly, 10^4 prestimulated T cells were cultured for 3 days with 10^5 irradiated strain 2 or 13 blood MNC (as a source of MHC-matched or mismatched antigen-presenting cells) and VZV or control antigens (prepared from VZV-infected and control Hartley fibroblasts as described by Zaia et al. [26]), and then their thymidine uptake during a 4-h pulse was determined.

Cytotoxicity assays. Effector lymphocytes were adjusted to $10^6/\text{ml}$ in RPMI 1640 with 10% fetal calf serum. Target cells were strain 2 L2C cells (which are strain 2 in origin but share class I antigens with strain 13) to which 10^4 PFU of VZV was added 16 h, before the cytotoxic assay. These cells showed no morphological evidence of cytopathic effect when harvested. In certain experiments, strain 2, strain 13, Hartley, and human fibroblasts were used as targets. These fibroblasts were VZV infected 72 h before harvesting. Target cells were labeled with $100 \mu\text{Ci}$ of ^{51}Cr for 40 min at 37°C and washed three times before being resuspended to 5×10^4 cells/ml. One hundred microliters of effectors was mixed in a V-bottom well with $100 \mu\text{l}$ of targets, and the cells were briefly centrifuged before being incubated for 5 h at 37°C . Then $100 \mu\text{l}$ of culture supernatant was aspirated for gamma counting. Percent specific lysis was expressed as [(experimental release-spontaneous release)/(maximum release-spontaneous release)] $\times 100$.

TABLE 1. Proliferative responses of guinea pig lymphocytes to VZV^a

Animal (no. of weeks)	Thymidine uptake ^b with in vitro stimulus		
	Control	VZV-Ag	VZV-Ag + anti-Ia
Control (1)	280 $\pm$ 70	360 $\pm$ 120	300 $\pm$ 90
VZV (1)	385 $\pm$ 100	330 $\pm$ 90	270 $\pm$ 70
Control (2)	250 $\pm$ 80	350 $\pm$ 60	300 $\pm$ 90
VZV (2)	280 $\pm$ 70	900 $\pm$ 100	320 $\pm$ 120
Control (4)	260 $\pm$ 20	155 $\pm$ 60	200 $\pm$ 100
VZV (4)	190 $\pm$ 70	1,592 $\pm$ 80	210 $\pm$ 40

^a Blood lymphocytes from strain 2 guinea pigs, either control or after injection with VZV, were cultured with a 1:200 dilution of VZV antigen. Thymidine uptake was determined after 6 days of culture.

^b Results are the mean $\pm$ 1 standard error of triplicate cultures for two strain 2 animals at each time point.

Cross-priming. Two strain 13 guinea pigs were injected intraperitoneally with 20 ml of mineral oil. Seven days later, their ascites was tapped and the peritoneal exudate (PE) cells were recovered by centrifugation. PE cells (2×10^7) were incubated in siliconized containers with an equal number of VZV-infected strain 13 guinea pig fibroblasts in RPMI 1640 medium with 5% guinea pig serum for 6 h, after which the cells were washed and irradiated with 2,000 rad. These cells were then injected intraperitoneally into strain 2 and 13 guinea pigs. After 20 days, blood and lymph node MNC were recovered from the injected animals and cultured with glutaraldehyde-fixed control or VZV-infected strain 13 fibroblasts. After 7 days, the MNC were recovered and tested for lysis of L2C cells (control or VZV infected).

RESULTS

Timing and phenotype of VZV-specific immune response in guinea pigs. Guinea pigs were injected subcutaneously with VZV which had been grown in guinea pig fibroblasts of the corresponding strain. Blood was obtained at 1-week intervals and tested for antibody by Western blot. Serum antibody was detected as early as 11 days following injection and included antibody with specificity for gpI. Proliferative responses by blood lymphocytes were detected 3 weeks after injection (Table 1). VZV-specific proliferation was blocked by the 22C4 antibody, as previously reported for other antigens (9). Proliferating cells in these cultures were identified by their blastlike light-scattering qualities and were found to stain with the 155 antibody, which defines a CD4-like subset of guinea pig T lymphocytes (Table 2). The blasts did not stain with CT6.

APC requirement for T-cell response to VZV. Blood MNC from VZV and control immunized guinea pigs were stimulated with VZV in the form of VZV-infected or control strain-matched fibroblasts in the presence or absence of adherent cells. The results (Table 3) show that proliferative responses required the presence of strain-matched antigen-presenting cells (APC) and was not influenced by the source of the infected fibroblasts.

As an additional check of the MHC restriction of VZV-specific responses, we examined the specificity of cytotoxic responses. Strain 2 and 13 guinea pigs were inoculated with VZV and their blood and lymph node lymphocytes were obtained 3 to 4 weeks later. These cells were stimulated for 7 days with VZV-infected autologous fibroblasts which had

TABLE 2. Phenotype of T-cell blasts from VZV-stimulated cultures^a

Animal	Stimulus	No. of weeks postinjection	% Cells stained			
			Small lymphocytes		Blasts	
			CD4	CD8	CD4	CD8
Control	Control	4	70	20	4	1
Control	VZV-stim ^b	4	72	18	3	1
VZV	VZV-stim	4	74	12	78	1
VZV	VZV-stim	8	72	8	77	1

^a Blood lymphocytes obtained from guinea pigs 4 or 8 weeks after injection with VZV were stimulated for 6 days in vitro with MHC-matched control or infected fibroblasts, after which the cells were recovered and stained with the CD4-like antibody, 155, or the CD8-like antibody, CT6.

^b stim, Stimulator cells.

been lightly fixed with glutaraldehyde. This degree of fixation does not interfere with class I-restricted antigen recognition by mouse T cells (17, 24). Culture was continued for a further 7 days after the addition of 10 U of human IL-2 per ml. Strain 2 effector cells recovered from these cultures lysed VZV-preincubated, but not control, L2C cells, and the lysis was inhibited with anti-MHC-II monoclonal antibody (Table 4). The lack of lysis of VZV-L2C cells by the strain 13 effectors (which have class I MHC antigens in common) suggests that class I-restricted cytotoxicity did not reach detectable levels in this system. Attempts to demonstrate class I-restricted cytotoxicity using VZV-infected strain 2 and 13 and xenogeneic fibroblasts as targets were unsuccessful because the IL-2-precultured guinea pig MNC lysed fibroblasts regardless of antigen specificity.

Cross-priming to elicit class I-restricted cytotoxicity. A possible explanation for our failure to detect class I-restricted VZV-specific cytotoxicity by MNC from VZV-inoculated guinea pigs was that our choice of target cells, or the use of an in vitro culture period, favored CD4 responses. To address this possibility, we used the cross-priming ap-

TABLE 3. Proliferation of lymphocytes from VZV-injected guinea pigs requires accessory cells but not MHC-matched stimulator cells^a

Strain and treatment ^b	Thymidine uptake with stimulator cell origin:			
	Strain 2		Human	
	Control	VZV	Control	VZV
2 (0, 0)	180 ± 30	210 ± 80	190 ± 70	170 ± 80
2 (0, +)	320 ± 120	270 ± 90	320 ± 90	250 ± 80
13 (0, 0)	300 ± 100	250 ± 60	330 ± 120	200 ± 90
13 (0, +)	390 ± 130	300 ± 100	400 ± 130	340 ± 90
2 (+, 0)	90 ± 40	160 ± 70	120 ± 70	210 ± 70
2 (+, +)	110 ± 20	5,880 ± 390	200 ± 60	4,560 ± 110
13 (+, 0)	120 ± 90	130 ± 30	130 ± 50	120 ± 70
13 (+, +)	290 ± 90	3,780 ± 300	200 ± 80	3,970 ± 290

^a Blood lymphocytes from guinea pigs of the strain shown, either control or 4 weeks after VZV injection, were cultured with glutaraldehyde-fixed MHC-matched or xenogeneic (human) fibroblasts, either VZV-infected or control. Thymidine uptake was determined after 6 days of culture. Results are the mean counts per minute ± 1 standard error for triplicate cultures from three animals of each strain.

^b The first symbol in parentheses indicates VZV injection; the second symbol indicates APC addition (5×10^4 2,000-R-irradiated APC per well). 0, None; +, done.

TABLE 4. Cytotoxicity by guinea pig lymphocytes^a

Strain (stimulus)	% Specific lysis						
	L2C target			Fibroblast target			
	L2C-control	L2C-VZV	L2C-VZV + anti-Ia	Strain 2 control	Strain 2 VZV	Human control	Human VZV
2 (control)	8	7	7	7	8	12	11
2 (VZV)	8	32	12	10	15	11	14
13 (control)	7	9	9	2	3	4	3
13 (VZV)	9	7	8	4	15	20	20

^a Blood lymphocytes from VZV-injected animals were cultured for 7 days with uninfected (control) or VZV-infected fibroblasts of the corresponding MHC, after which the medium was replaced with fresh medium containing 10 U of recombinant human IL-2 per ml. After a further 7 days, the cells were recovered and added in 20:1 effector/target ratio to ⁵¹Cr-labeled control or VZV-infected (VZV) strain 2 or human fibroblasts. Percent specific lysis was determined after 4 h of incubation.

proach of Bevan (3) to elicit class I-restricted responses by injecting antigen on MHC-mismatched APC. We immunized guinea pigs with strain 13 PE cells which had been preincubated for 4 h with unfixed, VZV-infected or uninfected fibroblasts. We previously found this to be sufficient time for VZV antigens to be processed by human APC (23). Strain 13 PE cells were selected so that we could use the L2C cells as targets (effector and target cells had to be matched for MHC to avoid allospecific cytotoxicity). Blood and lymph node MNC from the strain 2 and 13 recipients were obtained 3 weeks after immunization and were stimulated in vitro with VZV-infected strain 13 fibroblasts for 1 week, followed by 1 week of expansion in IL-2. Immunofluorescent staining of the blast cells recovered from these cultures showed that approximately equal numbers bound the 155 and CT6 monoclonal antibodies, suggesting that at least some class I-restricted T cells might be responding. The effector cells recovered from the cultures of strain 2 lymphocytes lysed VZV-preincubated, but not control, L2C targets (Table 5). This lysis was not blocked by the anti-class II MHC antibody, although the same antibody had effectively blocked proliferation (Table 1) and VZV-specific lysis (Table 4). The failure of the anti-MHC class II antibody to block makes it unlikely that the lysis observed in the cross-priming combination was restricted by the strain 2 class II MHC (as might have occurred if VZV had been reprocessed in vivo from the strain 13 macrophages onto autologous APC). A simpler explanation is that the cross-priming strategy had indeed elicited a cytotoxic response which was restricted by the class I MHC antigens shared between strain 2 and 13 guinea pigs. The strain 13 effectors, by contrast, lysed the VZV-

TABLE 5. Cross-priming elicits class I-restricted cytotoxicity by guinea pig MNC^a

Strain of effectors	PE cell incubation	% Specific lysis of cells		
		L2C uninfected	L2C-VZV	L2C-VZV + anti-Ia
2	Uninfected	10	10	11
2	VZV	10	31	29
13	Uninfected	12	9	10
13	VZV	9	18	16

^a Guinea pigs of the strain indicated were immunized once with 5×10^7 strain 13 PE cells which had been preincubated with strain 13 fibroblasts, either uninfected or VZV infected. Three weeks later, MNC were stimulated in vitro and tested for lysis of L2C targets.

infected L2C cells less well than the strain 2 effectors. The lack of suitable target cells prevented us from determining whether these animals had VZV-specific cytotoxic cells restricted by the strain 13 class II MHC.

DISCUSSION

Guinea pigs, inbred and outbred, develop T-cell responses to VZV, and the 3-week interval between inoculation and *in vitro* proliferation by T cells we found is consistent with previous reports (2, 18). The guinea pig strains we used (2 and 13) share class I MHC antigens but differ at both class II MHC loci. We extend previous observations by showing that VZV-stimulated proliferation by guinea pig lymphocytes requires the presence of strain-matched APC (such as irradiated blood MNC) even when the stimulating infected fibroblasts share MHC class I antigens with the responders. This observation, taken together with the ability of VZV-specific guinea pig T cells to proliferate in response to xenogeneic VZV-infected fibroblasts and autologous APC, suggests that the proliferative response is to VZV antigens which are re-presented on APC. The requirement for autologous APC implicates the class II MHC antigens, as these are the only antigens at which strains 2 and 13 are known to differ. The CD4-like phenotype of the proliferating T cells is generally consistent with this interpretation, although guinea pig leukocyte phenotypes are not as securely established as those of humans.

A critical question was whether our failure to detect class I MHC-restricted proliferative responses in the conventionally inoculated animals was an artifact of the assay systems or arose from the *in vivo* handling of the virus. The results of the cross-priming experiment are reassuring because they indicate that class I-restricted responses can indeed be detected in our assay system. The mechanism of the response to cross-priming is not clearly elucidated, but a plausible explanation for our results would be that the strain 2 animals responded to the MHC class I-associated VZV on the strain 13 APC, and that this response was more readily detected because there were no shared class II antigens to permit a class II-restricted response. The cross-priming experiment could be performed only in the direction of strain 13 macrophages into strain 2 animals because no strain 13 lymphoblastoid target cells were available.

Our data suggest that where the conditions of immunization with VZV mitigate against a class II-restricted response (because the virus-presenting cells do not have class II antigens in common with the responding cells), class I-restricted responses to VZV become detectable. Recently, VZV-specific human CD8⁺ T lymphocytes have been obtained from VZV-stimulated cultures by first depleting the cultured cells of the CD4⁺ subset by sorting. These data are consistent with the demonstration by Nash and coworkers that CD4 and CD8 responses to herpes simplex virus can function independently of each other in mice (22). VZV is reported to remain latent in dorsal root neurones (12) or their surrounding satellite cells (10). The neurones express only MHC class I antigens, while satellite cells constitutively express class II as well as class I antigens. Whether one or the other T-cell subset has a critical role in the maintenance of latency in either of these cell types remains to be determined.

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