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Specificity of the Blastogenic Response of Human Mononuclear Cells to Herpesvirus Antigens

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Peripheral blood mononuclear (PBM) cells from donors with a history of prior infection with herpes simplex virus, varicella-zoster virus, and/or cytomegalovirus were cultured for 2 to 8 days with glycine-extracted antigens prepared from these viruses and from infectious bovine rhinotracheitis virus. The proliferative response of PBM cells from all donors was specific during the first 6 days in culture. During this period the cellular immune responses of the seronegative donors were clearly different from those of the seropositive donors. The responses of PBM cells in culture with any of the human herpesvirus antigens studied was not influenced by prior infection of the donor with one or more other human herpesviruses. In contrast, although no donors had antibody to infectious bovine rhinotracheitis virus, the PBM cells from some of them had a proliferative response to this bovine herpesvirus, which increased with time. This nonspecific response appears to be a host-associated function which may be related to recognition of a common herpesvirus antigen.

The most commonly used test of cell-mediated immunity to human herpesviruses, herpes simplex virus (HSV), varicella-zoster virus (VZV), and cytomegalovirus (CMV), is the blastogenic response of peripheral blood mononuclear (PBM) cells to herpesvirus antigens. In normal persons a correlation has been demonstrated between prior infection with herpesviruses, as measured by the presence of specific antibody, and the in vitro response of PBM cells to antigens prepared from these viruses (3, 9, 10, 13-16). However, the interpretation of these results has generally ignored the potential of herpesviruses for stimulating a heterologous immune response (4, 11) This possibility was considered by Møller-Larsen et al. in their demonstration that the response of human PBM cells to purified CMV antigen was not influenced by preexisting immunity to HSV (7). In this report we measure the influence of prior infection with one or more of the three human herpesviruses on the specificity of the blastogenic response of human PBM cells to glycine-extracted antigens prepared from these viruses.

MATERIALS AND METHODS

Tissue culture. Antigens were prepared in human embryonic lung fibroblasts (HELF) obtained from J. Waner (Harvard School of Tropical Public Health) and used between passages 15 and 25. Cells were grown in Dulbecco-modified Eagle medium supplemented with 10% fetal bovine serum in 32-ounce (ca. 1-liter) glass bottles and were maintained in medium containing 2% serum.

CMV antigen. CMV (strain AD 169) was obtained from J. Waner and grown to a titer of 5×10^5 plaque-forming units/ml in HELF cells. Early confluent cultures of HELF cells were infected with cell-free CMV at a multiplicity of infection of 0.1 plaque-forming unit/cell. Cultures were harvested at 14 days.

VZV antigen. VZV (strain CP 5,262) was obtained from J. Nakano (Center for Disease Control, Atlanta, Ga.), and cell-associated virus was used at a multiplicity of infection of 0.1 focus-forming unit/cell. Cultures were harvested after 96 h.

HSV antigen. HSV, type 1 (strain VR 3), was obtained from E. Palmer (Center for Disease Control, Atlanta, Ga.) and used at a multiplicity of infection of 0.1 plaque-forming unit/cell. Cultures were harvested after 48 h.

IBR antigen. Infectious bovine rhinotracheitis (IBR) (strain LA) was obtained from the American Type Culture Collection and adapted to growth in HELF cells by three serial passages. HELF cells were infected at a multiplicity of infection of 0.1 plaque-forming unit/cell. Cultures were harvested after 48 h.

Method of harvesting. Infected cultures were harvested when cytopathic effect was observed in the entire monolayer. Uninfected cultures were harvested 4 days after mock infection to prepare control antigen. Cultures were decanted, washed twice with 10 ml of glycine-buffered saline (0.043 M glycine-0.15 M NaCl, pH 9.0), scraped into 5 ml of glycine-buffered saline, and homogenized at 4°C with a Dounce homogenizer. After storage at 4°C for 16 h, the homogenates were clarified by centrifugation at 600 × *g* for 10 min. Infectivity was eliminated from the preparation by irradiation for 15 min at a distance of 7.5 cm from a 30-W GE 30 T8 ultraviolet lamp. Irradiated preparations did not produce cytopathic effect in HELF or human embryonic kidney cells. The protein concen-

tration of the antigens ranged from 1,200 to 1,600 μg/ml as determined by the method of Lowry et al. (5). Antigen potency, as measured by complement fixation using standard antisera (2), was 640 U/ml for CMV, 320 U/ml for HSV and VZV, and 160 U/ml for IBR. All antigens were stored at −70°C.

Blastogenic assay. PBM were isolated from heparinized blood obtained from normal adult volunteers. Blood was diluted with Hanks balanced salt solution, layered over Ficoll-Hypaque gradients, and centrifuged at 400 × *g* for 30 min (1). PBM cells obtained from the interface were cultured at a concentration of 10^6 cells/ml in 1 ml of RPMI 1640 containing 10% pooled AB+ serum. The serum pool was obtained from male donors and contained antibody titers as follows: VZV (immunofluorescence assay), 4; HSV (complement fixation), 16; CMV (complement fixation), 8; IBR (complement fixation), <2. Six hours before harvest duplicate cultures received 1.0 μCi of [*methyl*-^{3}H]thymidine (20 μCi/mmol). After 6 h of labeling, triplicate 0.1-ml samples of the cultures were placed into wells of a microtiter plate and harvested with a multiple automated sample harvester onto fiber glass filters. Incorporation of radioactive thymidine into harvested cells was determined in a Beckman LS-335 scintillation spectrometer in 5 ml of Liquifluor (New England Nuclear).

Antibody assays. VZV-specific antibody to membrane-associated surface antigen was determined by the immunofluorescence assay (18). Antibody to HSV and CMV was determined by both complement fixation (2) and indirect hemagglutination (17). Antibody to IBR was determined by complement fixation and by neutralization (12). Standard antiserum to IBR was provided by L. Babiuk (Saskatoon, Saskatchewan, Canada).

RESULTS

Normal donors of PBM cells were chosen on the basis of prior experience with herpesviruses as determined by the presence of specific antibody (Table 1). Those studied included persons with antibody against one, two, or three of the human herpesviruses. One donor (MH) had no antibody against any of these viruses.

Preliminary experiments indicated that blastogenesis was stimulated by viral antigen over a broad range of dilutions (Table 2). Subsequent experiments were performed at a 1:100 antigen dilution. The proliferative response of donor PBM cells was determined on days 2 through 8 of incubation with viral antigen (Fig. 1). After incubation with VZV, HSV, or CMV antigens, the PBM cells of donors seropositive for these viruses actively proliferated from days 4 through 8, with the peak response generally occurring on day 6. In contrast, seronegative donors had no stimulation through day 4 and, thereafter, had gradually increasing and variable stimulation. On either day 5 or 6, the cellular immune responses of the seropositive and seronegative donors were clearly different. The mean incorporation of [^{3}H]thymidine by the seropositive group was 15 to 20 times greater than that of the seronegative group (Fig. 2). The maximum incorporation for any seronegative individual with any antigen was 587 cpm, whereas the minimum for any seropositive donor was 1,800 cpm with HSV and VZV antigens and 1,412 cpm with CMV antigens. At later culture times the difference between the two groups was blunted (Fig. 1, Table 3).

The CMV antigen preparation did not stimulate PBM cells as vigorously. Donors seropositive for CMV showed slightly lower levels of peak proliferation, whereas seronegative donors had somewhat more nonspecific stimulation on

TABLE 1. *Antibody profile of PBM cell donors*[a]

Donor	VZV		HSV			CMV		IBR	
	CF	IFA	CF	IHA-1	IHA-2	CF	IHA	CF	Neut
KL	<4	<2	64	≥8,192	2,048	128	256	<2	<4
PR	<4	<2	16	2,048	64	16	512	<2	<4
MV	<4	<2	16	8,192	256	64	4,096	<2	<4
MH	<4	<2	<8	<8	<8	<8	<8	<2	<4
DC	8	64	<8	<8	<8	<8	<8	<2	<4
GS	<4	32	<8	<8	<8	<8	<8	<2	<4
JA	16	128	16	2,048	2,048	<8	<8	<2	<4
RI	32	128	32	512	512	<8	<8	<2	<4
MJ	<4	16	16	512	32	<8	<8	<4[b]	<4
MA	4	128	<8	<8	256	32	64	<2	<4
PS	4	16	32	8,192	512	64	1,024	<2	<4
PL	4	128	64	512	32	64	1,024	<4[b]	<4

[a] Serum antibody from donors of PBM cells was determined by complement fixation (CF) and indirect immunofluorescence (IFA) for VZV, by CF and indirect hemagglutination (IHA) for HSV and CMV, and by CF and neutralization (Neut) for IBR. Antibody to HSV and CMV was determined by J. Stewart, Center for Disease Control, Atlanta, Ga. HSV type-specific antibody was determined by IHA for HSV type 1 (IHA-1) and HSV type 2 (IHA-2). Negative antibody titers are defined as <2 for CF to IBR, <4 for CF to VZV, <4 for IFA and Neut, and <8 for IHA. Titer is expressed as the reciprocal of the end point serum dilution.

[b] Serum was anticomplementary at a 1:2 dilution.

days 6 through 8 (Fig. 1). Nevertheless, the two groups could be readily distinguished on day 5 or 6. On day 5 the mean incorporation was 180 cpm by the seronegative group and 2,650 by the seropositive donors.

The specificity of the blastogenic response was determined by correlating the PBM cell reactivity with the antibody status of the donor (Table 3). In every case, on day 5 of incubation, the PBM cells from donors seronegative for a given herpesvirus failed to incorporate label when exposed to antigens prepared from that virus. When the donor was immune to two herpesviruses, there was no cellular response to antigen prepared from the third herpesvirus. However,

TABLE 2. *Dose response of PBM cells to herpesvirus antigens*[a]

Dilution factor[c]	[^{3}H]thymidine incorporation (cpm)[b]		
	VZV	HSV	CMV
10	2,583	3,991	1,425
50	5,022	5,164	3,900
100	5,295	6,013	5,090
250	5,155	ND[d]	4,850
500	4,588	5,549	3,123
1,000	4,032	3,715	ND

[a] PBM cells from three immune donors were cultured at 10^6 cells/ml for 6 days with VZV, HSV, or CMV antigen.

[b] Expressed as the mean per 0.1-ml triplicate sample from duplicate 1-ml cultures.

[c] Reciprocal of the final antigen dilution in the cell culture medium.

[d] ND, Not done.

after a week in culture, PBM cells from certain donors (Table 3, PR, DC, and MJ) demonstrated increasing nonspecific uptake of label even though the response to mock antigen remained negligible. This loss of specificity or cross-reactivity was observed in PBM cells only from a minority of donors.

In an attempt to understand the loss of specificity with prolonged incubation, and in seeking to develop a control antigen that more closely resembled the test antigens, we studied the response of PBM cells to IBR, a herpesvirus of cattle with tissue culture growth characteristics similar to those of HSV. IBR has no recognized antigenic cross-reactivity with human herpesviruses (8), and none of the donors had any memorable exposure to cows. All donors were seronegative for IBR antibody (Table 1). The IBR antigen was previously shown to stimulate blast transformation in immune bovine PBM cells. (L. Babiuk, personal communication). The response of human PBM cells to this herpesvirus antigen remained low during the first 4 days in culture (Fig. 3), but after this interval the PBM cells of three donors (MA, PS, and MJ) responded briskly. These three donors had prior infection with at least two human herpesviruses, but six other donors who had experienced multiple herpesvirus infections failed to respond to IBR.

DISCUSSION

Despite the frequent use of in vitro assays to determine cell-mediated immunity to herpesvi-

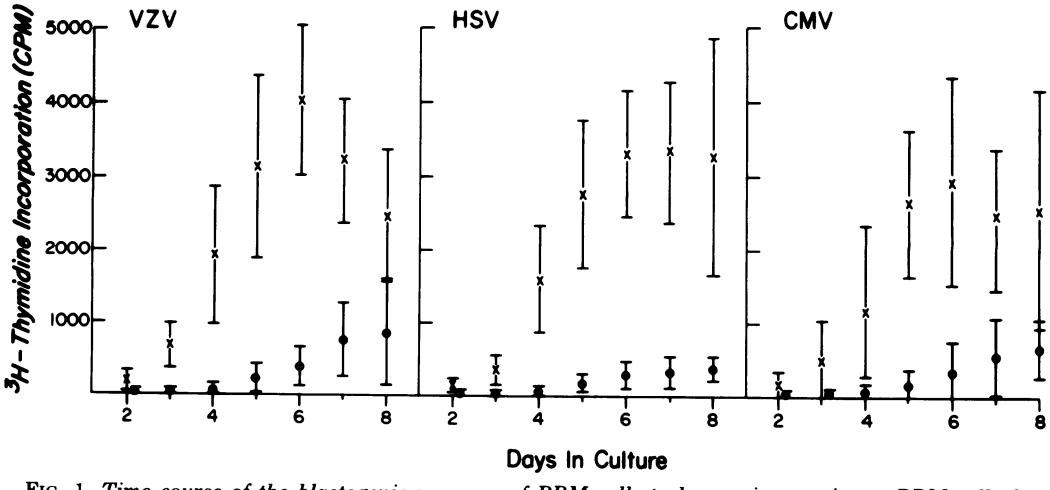

FIG. 1. *Time course of the blastogenic response of PBM cells to herpesvirus antigens. PBM cells from antibody-positive (×) or -negative (●) donors were cultured with antigens from VZV, HSV, and CMV. The immune status of the 12 donors is given in Table 1. [^{3}H]thymidine incorporation per 0.1 ml of cultured cells was determined on triplicate samples from duplicate cultures. The mean and one standard deviation for all the patients studied with each antigen are displayed.*

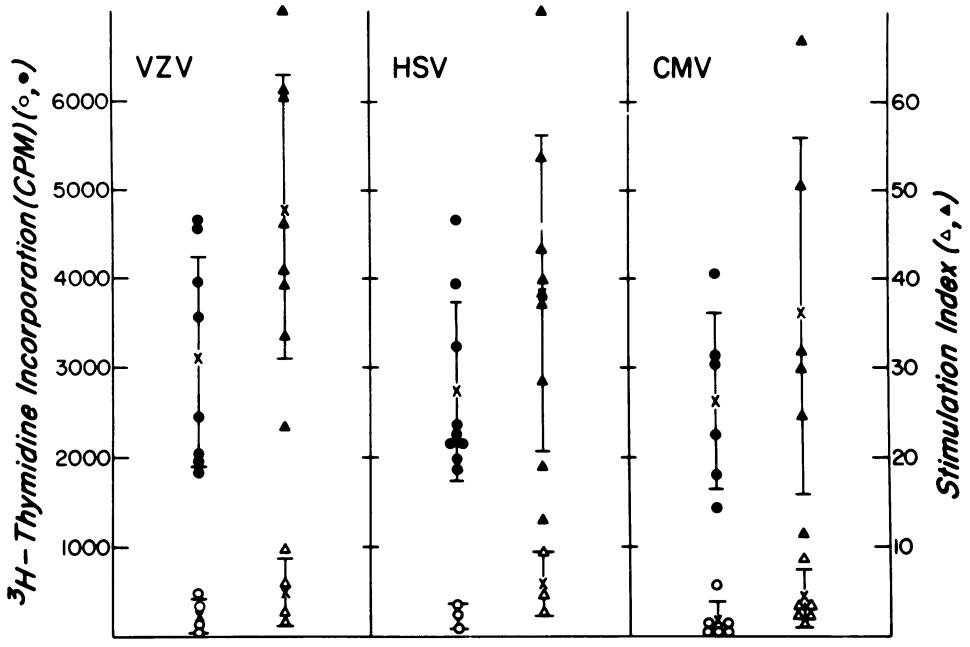

FIG. 2. *Day 5 blastogenic response to herpesvirus antigens by PBM cells from normal donors. PBM cells from antibody-positive (● or ▲) or -negative (○ or △) donors were cultured with antigens from VZV, HSV, and CMV. [^{3}H]thymidine incorporation per 0.1 ml of cultured cells (circles) was determined on triplicate samples from duplicate cultures. The stimulation index = (counts per minute incorporated with viral antigen)/(counts per minute incorporated with mock antigen) is given for the same donors (triangles). The mean (×) and one standard deviation are displayed.*

TABLE 3. *Specificity of the blastogenic response to herpesviruses*[a]

Donor				[^{3}H]thymidine incorporation (cpm) after 5, 6, or 7 days in culture											
Name	Antibody status[b]			VZV			HSV			CMV			Mock		
	VZV	HSV	CMV	5	6	7	5	6	7	5	6	7	5	6	7
KL	−	+	+	120	164	176	2,162	3,610	3,760	706	1,739	2,471	85	97	100
PR	−	+	+	255	720	1,517	2,001	3,261	4,306	3,158	3,023	3,761	105	156	340
MV	−	+	+	272	301	754	2,432	2,827	5,113	2,286	1,915	2,425	45	51	49
DC	+	−	−	1,760	3,271	1,978	367	665	727	96	236	1,331	38	127	161
GS	+	−	−	3,658	4,125	3,480	151	409	228	207	247	358	60	58	83
JA	+	+	−	2,105	3,381	2,316	3,328	3,842	3,393	139	281	179	88	140	231
RI	+	+	−	980	2,000	3,443	2,432	2,827	5,113	134	205	148	51	76	62
MJ	+	+	−	4,750	5,493	4,087	4,756	5,215	4,107	232	298	1,390	66	67	155

[a] PBM cells from donors were cultured with antigens prepared from VZV-, HSV-, and CMV-infected cells or uninfected cells (mock).

[b] Antibody-negative (−) or -positive (+) status is indicated; see Table 1 for quantitative serological characterization.

ruses, there has been little detailed analysis of the specificity of these reactions. Only a few studies have included a second herpesvirus as a control for cross-reactivity, even though it is known that human herpesviruses share cross-reacting antigens (4, 11). We have demonstrated that the blastogenic response of PBM cells exposed to HSV, VZV, and CMV antigens for 5 days is highly specific, and that the response of cells from seronegative and seropositive individuals is easily distinguished during this period (Fig. 1, Table 3). It is therefore apparent that prior infection with one or more herpesviruses does not influence the initial blastogenic response of individuals to herpesvirus antigens to which they are seronegative. However, begin-

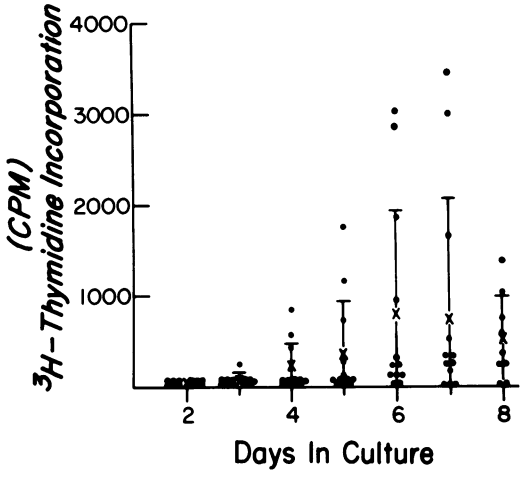

FIG. 3. *Blastogenic response of PBM cells to IBR. PBM cells from antibody-negative donors were cultured with IBR antigen. [^{3}H]thymidine incorporation per 0.1 ml of cultured cells was determined on triplicate samples from duplicate cultures. The mean counts per minute for each individual (●) and for the group (×) and one standard deviation of the group mean are displayed.*

ning on day 6, PBM cells from some antibody-negative donors became reactive to herpesvirus antigens to which they had not previously responded. We therefore recommend that the specific blastogenic response of PBM cells be determined on day 5 after addition of herpesvirus antigens. There was no unique combination of prior experience with herpesviruses which predicted those individuals who would respond nonspecifically to these viral antigens.

The apparent loss of specificity during extended culture could be due to delayed recognition in vitro of an antigen common to all herpesviruses or could represent in vitro sensitization of PBM cells from seronegative individuals. Consistent with the first possibility is the late response to IBR antigen observed in one-quarter of the donors tested (Fig. 3). None of the donors had antibody to IBR, nor did they have any unusual exposure to live cattle which could have resulted in IBR virus infection. Since it has recently been demonstrated that another bovine herpesvirus, bovine mammilitis virus, shares a common antigen with HSV, the possibility exists that a common antigen is also shared between human herpesviruses and IBR (6). The one donor without prior herpesvirus infection had no proliferative response to IBR through day 8.

The late loss of specificity could also be the result of non-immunologically mediated stimulation of cell division by some mitogen present in the viral antigen. However, such a mitogen would have to be present in each of three distinct herpesvirus antigen preparations and would have the peculiar property of stimulating PBM cells only from certain donors.

A further examination of any of these hypotheses will require herpesvirus antigens which are more pure than those we have used and may require antigens which do not include a cell membrane-associated component. Whatever the explanation, it is clear that only certain donors demonstrated a late response to viral antigens (human or bovine) for which they were seronegative, and that these donors could not be predicted on the basis of serum antibodies to herpesviruses. The nature of this host-related phenomenon merits further investigation.

Finally, we initially sought additional controls to study cell-mediated immunity to herpesviruses. IBR was chosen because this virus produces a cytopathic effect on human fibroblasts (HELF cells) which stimulates that produced by human herpesviruses (unpublished data) and because humans lack antibody to IBR. Figure 3 demonstrates that IBR antigen will not be the ideal negative control for assays of the human PBM cell response to human herpesviruses, since some donors are stimulated early to divide in response to IBR antigen.

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