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Stimulation of Peripheral Blood Lymphocytes by Herpes Simplex Virus in Vitro

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A method for stimulating sensitized lymphocytes by inactivated herpes simplex virus was established by using cultures of washed whole blood cells. The development of the immune response of herpes simplex virus-infected guinea pigs was examined at different times in the 4-month period after infection. Humoral and cellular immune responses were compared in individual animals by measuring lymphocyte stimulation ratios and neutralizing serum antibodies.

Considerable evidence from clinical data and animal studies points to the major role that cell-mediated immunity (CMI) plays in certain viral infections. CMI may particularly influence the development of the diseases and the recovery from the infections caused by the agents of the herpes- and poxvirus groups. Persistent and recurrent infections are especially common with the group of herpesviruses, where the presence of neutralizing serum antibody seems not to inhibit the recrudescence of the disease. Surprisingly few attempts have been made to evaluate the role of CMI versus humoral immunity in these infections. Indirect studies on the role of CMI examined the course of viral infections in thymectomized animals or in those treated with antilymphocyte serum (14, 19). However, in the absence of a suitable direct test, the development of the CMI in individual patients or in laboratory animals could not be followed in a manner similar to that used for studying the course of antivirus antibody production. Reactions of delayed-type hypersensitivity provide a sensitive technique for measuring CMI; however, this method has certain drawbacks. For instance, skin tests may sometimes be obscured by Arthus reactions. In addition, repeated skin testing in the same animal is not feasible since the skin test itself modifies the immune response, inducing or boosting the immune reaction of the animal. In vitro tests for CMI have recently been applied to measure the host's immune response to viral antigens (1, 8, 28-33, 38). Application of these techniques also has limitations, especially in small laboratory animals, since the animal usually has to be killed in order to obtain the cell material needed for the tests, and so again the development of the immune response can not be followed in the same animal.

Several authors described methods for the stimulation of peripheral blood lymphocytes without separation of the nucleated cells from the erythrocytes (4, 5, 10, 12, 17, 22-24). This technique needs minimal amounts of blood and enables one to follow the development of CMI in individual small laboratory animals. The subject of the present report is the applicability of this test for measuring CMI against herpes simplex virus (HSV) antigen in HSV-infected guinea pigs. In addition, the development of lymphocyte stimulation ratios and neutralizing antibodies were compared in individual animals.

MATERIALS AND METHODS

Immunization of animals. Male, white guinea pigs, strain Hartley, weighing 200 to 400 g, were immunized against HSV by infection with 10^5 plaque-forming units (PFU) of HSV into the two hind footpads. This inoculation gives rise to virus multiplication in the infected foot for several days, accompanied by an inflammatory reaction and the development of vesicles. Complete recovery occurs regularly about 10 days after infection, and no infectious virus can be demonstrated in the foot later.

Virus. HSV type 1, strain GJ, was isolated 3 years ago from a patient's herpetic lesions. Stock virus for infection of animals and for neutralization tests was grown in primary rabbit kidney cells in Eagle minimal essential medium without serum. Virus plaque titrations were performed in secondary chick fibroblasts under fluid overlay containing 0.2% of a rabbit anti-HSV hyperimmune serum.

Neutralizing antibodies. Neutralizing antibodies were determined in the presence of complement in microtiter plates (Microtest II, Falcon Plastics) by using BHK-21 cells.

Antigen for lymphocyte stimulation. Virus was grown in BHK-21 cells by using serum-free medium. At the time when the complete cytopathogenic effect had developed (usually 48 h after infection), the cells

were scraped into the medium and collected by centrifugation. The supernatant medium was used to prepare so-called "soluble" antigen (S-antigen), and virus-particle antigen (V-antigen) was prepared from the virus associated with the cytoplasm of the cells, as outlined below.

The cells were washed once in phosphate-buffered saline, pH 7.4, suspended in 1 mM phosphate buffer, pH 7.4, and disrupted by six strokes in a Dounce homogenizer. Sucrose was added immediately to give a 0.25-M final concentration to prevent leakage of nuclei. The suspension was partially clarified by low-speed centrifugation. A 14.5-ml amount of this virus suspension was layered on 3 ml of 60% sucrose and centrifuged in the SW27 rotor at 16,000 rpm for 90 min. The top solution was discarded, and the opaque virus band at the surface of the cushion was removed and dialyzed against 10 mM tris(hydroxymethyl)-aminomethane-hydrochloride buffer, pH 7.6, containing 2 mM ethylenediaminetetraacetate (Tris-EDTA). The material was then filtered through a membrane filter (0.45-μm pore size; Millipore Corp.), which had been treated before use by the method of Wallis and Melnick (37). Briefly, this involved passage of 5 ml of 10% inactivated guinea pig serum in Tris-EDTA through the filter, followed by 20 ml of serum-free buffer. This procedure increased the PFU-to-protein ratio of the preparation considerably, holding back virus aggregates as well as subcellular particles. The final virus suspensions contained between 10^6 and 2.10^7 PFU/ml and were heated at 56 C for 30 min to destroy infectivity. Suspensions were then put in ampoules and stored at −70 C.

The S-antigen was prepared by first concentrating the culture medium through an XM 20 ultrafilter (Amicon). The 10-fold concentrate was then clarified from particulate material by centrifugation at 50,000 $\times$ g for 90 min. The top half of the supernatant, containing S-antigen, was dialyzed against Tris-EDTA and sterilized by filtration through a 0.05-μm membrane filter (Millipore Corp.). It was stored in ampoules at −70 C.

Control antigens from noninfected BHK-21 cultures were prepared in a similar manner.

Blood-cell cultures. Blood was drawn by cardiac puncture into a heparinized plastic syringe. Whole-blood cultures and washed-blood-cell cultures were used. The medium used for whole-blood cultures was RPMJ 1640 containing 2 mM glutamine, 100 μg of streptomycin per ml, and 100 U of penicillin per ml. For the washed-blood-cell cultures, this medium was supplemented by 10% inactivated fetal calf serum.

Whole-blood cultures were set up without any manipulating of the heparinized blood. For washed-blood-cell cultures, the blood was centrifuged for 10 min at 250 $\times$ g, the plasma was drawn off, and the cells were washed once in 10 times the original volume of RPMI 1640–10% fetal calf serum. The cell sediment was then made up to the original volume with medium.

All cultures were prepared in round-bottom centrifuge tubes covered with Belco caps. To 0.1 ml of whole blood or washed blood cells, 0.8 ml of the respective medium and 0.1 ml of the antigen dilution were added. Cell concentrations for both types of cultures usually were between 2.5×10^5 to 5.0×10^5 nucleated cells per ml and around 10^8 erythrocytes per ml. All cultures were set up in triplicate and incubated at 37 C in an atmosphere of 5% CO_2 in air.

Determination of deoxyribonucleic acid synthesis. Blood-cell cultures were labeled with 1 μCi of [^{3}H]thymidine (Radiochemical Center, Amersham) 18 h before termination. A 4-ml amount of 3% acetic acid in saline was then added to the cultures to lyse the red cells. After centrifugation, the cell sediment was washed with phosphate-buffered saline, pH 7.4, and then the remaining hemoglobin was oxidized by treatment with 2 drops of 30% H_2O_2 for 5 to 10 min. High-molecular-weight material was then precipitated with 4 ml of 10% ice-cold trichloroacetic acid, and the precipitates were washed once in cold methanol, dissolved in 0.5 ml of hyamine hydroxide, and transferred into scintillation vials with 10 ml of scintillation fluid. Radioactivity was determined in a Packard liquid scintillation counter. Stimulation ratios were expressed as mean counts per minute of three replicates from stimulated cultures divided by the counts per minute of unstimulated controls.

Protein. Protein was determined according to the method of Lowry et al. (18).

RESULTS

Comparison of whole-blood cultures with washed-blood-cell cultures. Several other authors have established lymphocyte stimulation tests with whole-blood cultures, omitting supplementation of the medium by serum. This method has been compared with the washed-cell culture technique in this study also (Table 1).

Stimulation ratios of whole-blood cultures set up soon after immunization usually were slightly higher than the ratios obtained in washed-blood-cell cultures. However, late after immunization, whole-blood cultures tended to give considerably lower ratios, indicating that the amount or type of antibody contained in the late cultures could influence the result.

In contrast, there was no significant difference of stimulation between cultures of blood cells washed once, twice, or three times. To establish further the suppressive effect of antibodies on the lymphocyte response to the antigen, washed-blood-cell cultures were set up in a medium supplemented by either 10% normal guinea pig serum or by 10% of an anti-HSV hyperimmune serum from guinea pigs. The addition of specific antibody to the culture medium reduced the stimulation ratios (Table 2). Thus, for all further tests, once-washed cells were used.

Dependence of stimulation ratios on antigen dose and incubation time. To determine the optimal antigen concentrations for lympho-

TABLE 1. *Comparison of stimulation ratios obtained in whole-blood and washed-blood-cell cultures*

Time	Antigen dilution	Whole-blood cultures		Washed-blood-cell cultures	
		Counts/min	Stimulation ratio	Counts/min	Stimulation ratio
22 days postinfection		1,893	1	666	1
(neutralizing serum	1:100	9,269	4.9	750	1.1
antibody, 1:30)	1:200	12,523	6.6	924	1.4
	1:400	10,696	5.7	649	0.9
133 days postinfection		131	1	191	1
(neutralizing serum	1:20	203	1.6	2,664	13.9
antibody, 1:160)	1:60	893	6.8	18,591	97.3
	1:180	1,311	10.0	16,568	86.7
	1:540	1,580	12.0	12,278	64.3

TABLE 2. *Effect of anti-HSV hyperimmune serum on the stimulation ratios of washed-blood-cell cultures*

Antigen dilution	Stimulation ratio in cultures:	
	With antiserum	Without antiserum
	1	1
1:50	12.1	27.3
1:200	36.2	111.5
1:800	15.1	72.1

cyte stimulation and the optimal time of harvesting the cultures, blood-cell cultures were set up with various antigen concentrations and harvested after 4, 5, 6, and 7 days (Table 3).

Cultures harvested on day 4 or 5 gave relatively low stimulation ratios over a broad range of antigen doses. Examination of the cultures after 6 or 7 days always resulted in considerably higher stimulation ratios with an optimal antigen concentration that usually was higher than the optimal concentration for cultures harvested earlier. The optimal antigen concentration varied between animals within dose ranges of ±100%. Consequently, for routine tests at least three antigen dilutions in twofold steps were used.

HSV antigen preparation. V-antigen, prepared from the cell-associated virus, was compared with S-antigen, prepared from the medium. Both antigens were adjusted to the same protein concentration. The stimulating capacity of various dilutions of the two antigens was estimated in parallel in cultures of blood cells from six different HSV-infected animals. The V-antigen yielded stimulation ratios between 15 and 71 in cultures from five out of six animals, whereas the S-antigen gave rise to stimulation only in cultures from two out of the six guinea pigs, with ratios between three and six. Thus, in all further experiments only V-antigen was used.

In blood-cell cultures from noninfected animals, virus antigen as well as control antigen occasionally stimulated [^{3}H]thymidine uptake to threefold above the control values. Therefore, only ratios of fourfold or higher were considered positive for immunologically specific stimulation.

Development of the immune response in HSV-infected guinea pigs. The development of the immune response after HSV infection was measured by lymphocyte stimulation ratios and titers of neutralizing antibodies. Two groups of four animals infected simultaneously were tested alternately. From each animal, 5 ml of blood was drawn. The guinea pigs tolerate this blood donation every 2 weeks without any obvious detrimental effect. Drawing blood more often, however, leads to a strong tendency of erythrocytes to hemolyze in cultures and to inconsistent results in lymphocyte stimulations. Cultures were set up from the blood cells of each animal with three concentrations of the HSV antigen plus the corresponding three concentrations of control antigen and unstimulated control cultures. The HSV antigen concentrations used contained an amount of inactivated virus corresponding to 5×10^3 to 33×10^3 PFU of infectious virus per ml before heat inactivation (2.4 to 16 μg of protein per ml). Triplicates from each set were harvested on days 6 and 7 after onset.

Table 4 presents the maximal stimulation ratios of each set of cultures, irrespective of antigen concentration and incubation time yielding this value. It is noteworthy that the highest lymphocyte responses were obtained approximately as often in cultures terminated after 6 days as in those harvested after 7 days of incubation. Neither the antigen concentration nor the time of harvest that gave the highest ratio showed any correlation to the time after immunization. Thus, to obtain maximal responses, various antigen concentrations were

always used and isotope uptake was determined after at least two different incubation times.

Postitive stimulation (ratios >4) could be obtained as early as 7 days after infection (one out of four animals). All of the guinea pigs had positive reactions 3 weeks after infection. However, there were considerable variations of the lymphocyte stimulation among the individuals within the group of animals. For example, although one animal developed its highest response only 14 days after infection, most of the guinea pigs reached peak levels between 63 and 70 days after sensitization. No correlation could be demonstrated between titers of neutralizing antibodies (Table 5) and stimulation ratios. Furthermore, in an individual animal, humoral and cellular immune reactions did not always develop in parallel.

DISCUSSION

Stimulation of peripheral blood lymphocytes by antigen was shown to be correlated with cutaneous delayed hypersensitivity in several animal species including guinea pigs (13, 16, 20, 25, 27). Nevertheless, the secondary response of immune lymphocyte to an antigen in vitro does not exclusively reflect T-cell reactivity. In certain systems at least, stimulation of B lymphocytes may occur in addition (1, 6, 7). However, the test is usually considered primarily to determine CMI and has been applied and recommended recently by several authors to study CMI in viral infections (1, 5, 8, 28-33, 38). In the experimental HSV infection studied here, development and degree of lymphocyte responsiveness was apparently not correlated with the humoral antibody response. Thus, it can be assumed that the reactivity of the lymphocytes to the HSV antigen in vitro is equivalent to CMI against the infectious agent in the animal.

This report demonstrates that cultures of whole blood cells, instead of the conventionally used buffy-coat cells, can be used to examine the lymphocyte response against a virus antigen. This technique has the advantage of requiring about 1/10 the amount of blood needed for cultures of nucleated cell-enriched preparations (23; this report), allowing large-scale studies on CMI in small laboratory animals. Thus, with blood drawn from one guinea pig, detailed analyses of the parameters of the test system

TABLE 3. *Dependence of stimulation ratios upon antigen concentration and time of harvest of the cultures*

Antigen dilution	Antigen protein (μg/ml)	Day of harvest after onset of cultures							
		4		5		6		7	
		Counts/min	Stimulation ratio	Counts/min	Stimulation ratio	Counts/min	Stimulation ratio	Counts/min	Stimulation ratio
		747	1.0	535	1.0	108	1.0	191	1.0
1:20	9.10	3,013	4.0	2,977	5.6	588	5.4	2,664	14.0
1:60	3.03	3,850	5.2	4,318	8.1	5,321	49.3	18,591	97.3
1:180	1.01	4,695	6.3	4,974	9.3	4,261	40.2	16,568	86.7
1:540	0.34	3,506	4.7	3,384	6.3	2,624	24.3	12,278	64.3
1:1,620	0.11	4,721	6.3	3,588	6.7	2,467	22.8	10,812	56.6
1:4,860	0.04	4,975	6.7	2,893	5.4	2,902	26.9	9,659	50.6

TABLE 4. *Stimulation ratios of blood lymphocytes from HSV-infected guinea pigs at various times after infection*

Animal	Day after infection[a]									
	0	7	14	21	28	35	42	63	70	126
1499	3.3 (2.0)									
1497	2.9 (0.9)		2.9 (1.6)		?[b]		15.6 (1.6)		51.7 (0.8)	
1498	1.5 (0.8)		132.1 (1.3)		10.7 (0.8)		24.5 (0.6)		20.5 (0.9)	
1500	2.7 (0.7)		7.4 (1.0)		20.3 (0.5)		51.0 (0.8)		90.3 (1.1)	
1491			5.4 (0.4)		14.0 (2.3)		43.4 (0.6)		4.8 (2.8)	
1495		1.1 (0.9)								
1493		3.9 (1.4)		7.8 (0.1)		35.5 (2.3)		106.3 (0.8)		3.3 (0.5)
1494		13.3 (1.8)		16.2 (0.8)		14.5 (1.3)		184.0 (1.4)		8.5 (1.1)
1496		3.3 (1.5)		42.3 (1.1)		72.1 (1.2)		16.6 (2.1)		9.7 (0.6)
1646				5.9 (2.5)		15.5 (0.7)		18.1 (0.8)		11.2 (0.7)

[a] Numbers represent the highest stimulation ratios obtained with HSV antigen; numbers in parentheses indicate the values obtained with the corresponding concentration of the control antigen.

[b] Irregular [^{3}H]thymidine uptake caused by strong hemolysis of the cultures.

TABLE 5. *Neutralizing antibody titers in HSV-infected guinea pigs at various times after infection*

Animal	Day after infection[a]									
	0	7	14	21	28	35	42	63	70	126
1499	<5									
1497	<5		17.5		35		80		NT[b]	
1498	<5		40		40		30		NT	
1500	<5		NT		60		120		NT	
1491			17.5		40		100		NT	
1495		NT								
1493		NT		50		80		160		70
1494		12.5		NT		30		40		30
1496		NT		60		30		200		320
1646				35		80		120		120

[a] Numbers are reciprocals of serum dilution.
[b] NT, Not tested.

may be accomplished. Moreover, the immune response during the course of infection may be followed in individual animals by repeated tests.

Whole-blood cultures have been applied previously for lymphocyte stimulation by mitogens (4, 10, 12, 17, 22–24) or various antigens (5, 17, 23, 24). Most of the authors recommend methods which consist of simply diluting the anti-coagulated blood in serum-free medium. However, the influence of antibodies in the plasma should be taken into account when lymphocytes of immunized animals are to be stimulated by the respective antigen. Antigen-antibody complexes which may form in such cultures are capable of modifying the response of the lymphocytes to the antigen. Depending on the antigen-antibody ratios in the system, the antibodies may increase or impair the antigen-induced thymidine uptake of the lymphocytes (1, 3, 11, 21, 31, 36). Surprisingly, several authors using whole-blood cultures (5, 17, 24) or recommending the use of autologous plasma in pure lymphocyte cultures (29, 32, 33) either did not observe or did not mention an antibody-mediated influence on the test. In the case of viral antigens, Gerber and Lucas (8) and Rosenberg et al. (28) claimed that the addition of specific antibodies did not alter the lymphocyte reaction toward the antigen. The present study clearly is at variance with these results. Early after infection, whole-blood cultures showed responses similar to, or slightly higher than, washed-blood-cell cultures. Late after infection, however, when antibodies of high titer and probably high avidity were present in the plasma, the stimulation ratios of the washed-blood-cell cultures were considerably higher than those of the whole-blood cultures. In addition, the stimulation ratios of washed-blood-cell cultures could be reduced by the addition of anti-HSV antibodies. It was concluded, therefore, that when lymphocyte reactivity is to be compared at different times after immunization, the cells should be washed free of antibodies.

It has been reported that in order to demonstrate cutaneous delayed hypersensitivity against HSV, the S-antigen is a more sensitive indicator than the V-antigen (2, 15). Therefore, both types of antigen were tested for in vitro lymphocyte stimulation. In accordance with the results of Rosenberg et al. (28), V-antigen was found to yield more frequently positive and considerably higher stimulation ratios. However, since both antigens are far from being pure or biochemically characterized, the different sensitivity of the two tests to the two types of antigen does not prove that different immune responses of the host are measured by the in vivo and in vitro test for CMI.

The development of blood lymphocyte reactivity toward HSV antigen could be followed by the described method in eight guinea pigs between 0 and 126 days after infection. Two observations made during this study should be particularly stressed. (i) The degree of stimulation as well as the time of peak response varied considerably between animals. This wide variability strongly emphasizes the value of a test by which CMI can be followed in individual animals. (ii) Previous reports on the time course of lymphocyte reactivity toward viral antigen in experimentally infected animals demonstrated a short-lived peak response soon after infection, which dropped to low levels after some days (5, 9, 28). In contrast to these observations the lymphocytes of HSV-infected guinea pigs showed a comparatively late onset of responsiveness. The degree of stimulation, however, increased in most animals over several weeks,

and high levels were maintained over a rather long period of time. The titers of neutralizing antibodies showed the same long-term increase and did not reach top levels until 63 to 70 days after infection. The time course of both immune responses suggests that, after the acute and self-limiting local infection, the HSV may persist in the guinea pigs, constantly boosting the immune response. Persistence of HSV in sensory ganglia of men and experimentally infected rabbits and mice has been reported (26, 35). In HSV-infected guinea pigs, persistent virus could also be demonstrated in spinal ganglia up to at least 200 days after infection (M. Scriba, unpublished data), which may account for the long-lived lymphocyte reactivity.

Reactivation of latent herpesviruses or reinfection are not inhibited by neutralizing serum antibodies. On the other hand, HSV recrudescence may well be related to impairment of CMI. The lymphocyte stimulation test described here offers an easily performed and reliable method to study the role of CMI in HSV infections of men and experimental animals.

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