***In Vitro* Stimulation of Sensitized Lymphocytes by Herpes Simplex Virus and Vaccinia Virus**

(cellular immunity/humoral immunity/blocking antibody/peripheral leukocytes/viral antigens)

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ABSTRACT Splenic lymphocytes from rabbits immunized with herpes simplex virus (HSV) were incubated *in vitro* with ultraviolet light-inactivated HSV, and the degree of lymphocyte transformation was determined by measurement of the incorporation of [³H]thymidine into acid-insoluble material. Lymphocytes from immunized rabbits were stimulated as much as 30-fold, whereas lymphocytes from control rabbits were unaffected. Lymphocyte sensitization occurred within 3 days after immunization; sensitized lymphocytes could still be detected 120 days after immunization. The antigenicity of the ultraviolet light-inactivated crude virus pool was found to reside primarily in the virion. Infectious virus, in comparison with inactivated virus, produced less lymphocyte stimulation. Studies on the interaction of the humoral and cellular immune responses showed that incubation of anti-HSV antibody with HSV antigens did not reduce the capacity of the viral antigens to stimulate sensitized lymphocytes. Other experiments revealed that lymphocytes from both the spleen and peripheral blood of animals immunized with vaccinia virus could be stimulated by vaccinia, but not by HSV. Conversely, lymphocytes from animals immunized with HSV could not be stimulated by vaccinia. The transformation of sensitized lymphocytes by viral antigens appears to be a simple, highly specific, and quantitative *in vitro* technique for the study of the cellular immune response to viral infections.

Cell-mediated immunity is thought to be an important factor in the defense against certain viral infections (1), but its precise role has been difficult to assess because of the lack of a simple *in vitro* assay. A number of *in vitro* systems, however, have been developed and are widely used for the study of the cellular immune response to nonviral antigens (2, 3). Recently, studies from several laboratories suggest that lymphocytes from specifically immunized animals will respond to viral antigens *in vitro*, as measured by blastogenesis, migration inhibitory factor production, and cytotoxicity (4-11). There is, however, little if any quantitative information on the preparation and nature of the viral antigens, the time of appearance and magnitude of the immune response, and the specificity of the reaction. The present report on virus-induced lymphocyte stimulation describes a simple and quantitative *in vitro* system for the study of the cellular immune response to viral antigens, and provides some information on the factors and conditions involved in virus-lymphocyte interaction.

Abbreviations: HSV, herpes simplex virus; PBS, phosphate-buffered saline; PFU, plaque-forming units.

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MATERIALS AND METHODS

Virus Preparations. Herpes simplex virus (HSV; type 1) and vaccinia virus (strain CVI-79) were grown and assayed in primary rabbit kidney cells (12, 13). The stock pools of crude HSV and vaccinia contained 7.5×10^7 plaque-forming units (PFU)/ml and 4.0×10^6 PFU/ml, respectively. Partially purified virus was prepared by centrifuging the stock virus pools at $78,500 \times g$ for 60 min in a Beckman model L ultracentrifuge. The supernatant fluid was used as the source of soluble viral antigens; the viral pellet was suspended in phosphate-buffered saline (PBS, 0.02 M, pH 7.2), centrifuged at $78,500 \times g$ for 60 min, and then resuspended in PBS to one-half of the original volume. The titers of the partially purified pools of HSV and vaccinia were 1.5×10^8 PFU/ml and 7.5×10^6 PFU/ml, respectively. Density-gradient purified virus was prepared by centrifugation of partially purified virus at $100,000 \times g$ for 90 min on a 10-30% sucrose density gradient.

Immunization Procedures. 6- to 8-week-old male New Zealand White rabbits, were immunized with partially purified virus by the intravenous route (1.0 ml), the intradermal route (0.5 ml), or the corneal route (12). Except where indicated, rabbits were inoculated intradermally. Rabbits injected with crude or partially purified homogenate of cells from uninfected primary rabbit kidney culture were used as controls. Hyperimmune serum to HSV was prepared in rabbits by repeated intravenous injections of partially purified virus. The 50% plaque-neutralization titer of the serum based on 100 PFU of virus was 1/8000 (12).

Leukocyte Cultures. Growth medium for leukocyte cultures consisted of RPMI 1640 medium (NIH Media Unit) supplemented with 50 μ g of neomycin/ml, 100 units of penicillin/ml, 2 mM glutamine, and 10% fetal-calf serum (heated at 56° for 30 min). At various times after immunization, spleens were obtained aseptically from rabbits, trimmed of excess tissue, and minced with scissors. The mince was suspended in serum-free growth medium, passed sequentially through 16- and 19-gauge needles, and centrifuged at $50 \times g$ for 1 min. The pellet was discarded and the supernatant fluid was centrifuged at $250 \times g$ for 5 min. The cells were resuspended in growth medium at a density of 4.0×10^6 cells/ml. For peripheral blood leukocyte cultures, 5-20 ml of heparinized blood were obtained by cardiac puncture. One volume of heparinized blood and two volumes of a sterile solution of 2.5% Dextran in RPMI 1640 medium were mixed thoroughly

and incubated at 36.5° for 60–120 min to sediment the erythrocytes. The leukocyte-rich supernatant fluid was aspirated and centrifuged at $200 \times g$ for 5 min. The cells in the pellet were washed and suspended in growth medium at a density of $1.0\text{--}4.0 \times 10^6$ cells/ml. The cell suspensions were dispensed in 1-ml aliquots into sterile (1 dram) screwcap glass vials. Unless specified otherwise, spleens obtained 12 days after immunization were used in all experiments.

Culture Stimulants. Crude HSV (7.5×10^6 PFU/0.1 ml) and crude vaccinia virus (4.0×10^6 PFU/0.1 ml) were used routinely as the source of viral antigens. Partially purified virus, density gradient-purified virus, and soluble viral antigens were used only where indicated. All virus preparations, unless otherwise stated, were inactivated by exposure to ultraviolet light (UV) for 4 min at a distance of 15 cm. All UV-treated virus preparations were found to be negative for infectious virus. Conditioned medium and cell homogenate (frozen and thawed twice and clarified by centrifugation at $2000 \times g$ for 15 min) from uninfected primary rabbit kidney cultures were used as negative controls. To show that lymphocyte preparations were capable of being transformed *in vitro*, 6 μ g of phytohemagglutinin (Batch E 316/Q1 Burroughs-Wellcome, Tuckahoe, N.Y.), in 0.1 ml of unsupplemented medium was added to triplicate cell cultures in each experiment. In every case, 5- to 150-fold stimulation was observed after 72-hr incubation, as measured by the incorporation of [3 H]thymidine into acid-insoluble material (see below).

Transformation of Leukocyte Cultures. The incorporation of [3 H]thymidine into acid-insoluble material was used for measurement of the degree of lymphocyte transformation induced by viral antigens. In a typical experiment, 0.1 ml of undiluted test material was added to triplicate cell cultures, and the cultures were incubated at 36.5° for 72 hr in an atmosphere of 5% CO₂ and 95% air. 1 μ Ci of [3 H]thymidine (18 Ci/mmol New England Nuclear, Boston, Mass.); were added to each culture for the final 4 hr of incubation. The cells were then washed once with 3 ml of PBS, twice with 3 ml of 5% trichloroacetic acid, and once with 3 ml of absolute methanol. The resulting precipitates were dissolved at 60° for 15 min in 0.2 ml of NCS (Amersham-Searle Corp., Arlington Heights, Ill.), and 3 ml of Liquifluor-toluene (1:25) scintillation fluid was added (Liquifluor, New England Nuclear, Boston, Mass.). Samples were counted in a Packard

FIG. 1. Stimulation ratio at different times after addition of viral antigens to cultures of sensitized lymphocytes.

Tri-Carb liquid scintillation spectrometer, model 3375. For all data presented, values for triplicate cultures were within $\pm 10\%$ of their mean. Results are expressed either as the mean cpm of triplicate cultures or as the ratio (stimulation ratio) of cpm of [3 H]thymidine incorporated in the presence of the test material divided by cpm incorporated in the presence of conditioned medium or PBS.

RESULTS

Stimulation of sensitized lymphocytes by HSV

The ability of HSV to stimulate sensitized splenic lymphocytes is illustrated in Table 1. HSV antigens, conditioned medium, and cell homogenate were incubated with lymphocytes from both immunized and control animals. It can be seen that HSV induced a 10-fold stimulation of lymphocytes from immunized animals, but had no effect on lymphocytes from control animals. Cell homogenate had no stimulatory effect on lymphocytes from either group.

The next set of experiments was designed to study the conditions required to produce optimal stimulation of sensitized lymphocytes *in vitro*. Fig. 1 shows that lymphocyte stimulation could be detected within 24 hr after addition of viral antigens, reached a maximum at 72 hr, and declined over the next 48 hr. Other experiments showed that the degree of stimulation and time of maximal stimulation were dependent on the number of cells in culture. When the number of cells was reduced from 4.0×10^6 to 1.0×10^5 , maximum stimulation was not observed until 120 hr after addition of viral antigens.

The data in Fig. 2 show that the degree of lymphocyte stimulation is directly dependent upon the concentration of viral antigens. Undiluted HSV antigens (equivalent to 7.5×10^6 PFU before UV-inactivation) produced a 10-fold stimulation of sensitized lymphocytes, whereas a $1/8$ dilution

TABLE 1. *In vitro* stimulation of lymphocytes by HSV

Test material	Lymphocytes from HSV-immunized animals		Lymphocytes from control animals	
	[3 H]Thy- midine uptake (cpm)	Stimu- lation ratio*	[3 H]Thy- midine uptake (cpm)	Stimu- lation ratio
Conditioned medium	5,091	—	3,325	—
Cell homogenate	3,682	0.72	2,268	0.68
HSV	54,219	10.65	1,168	0.50

* Stimulation ratio = cpm in presence of test material/cpm in presence of conditioned medium.

FIG. 2. Effect of concentration of viral antigens on the stimulation of splenic lymphocytes. (●—●), sensitized lymphocytes plus HSV antigens; (○—○), control lymphocytes plus HSV antigens; (▲—▲), sensitized lymphocytes plus cell homogenate; (△—△), control lymphocytes plus cell homogenate.

produced only a 2-fold stimulation. Incubation of viral antigens with control lymphocytes or incubation of cell homogenate with sensitized or control lymphocytes failed to produce stimulation.

To determine whether soluble viral antigens or the virion was responsible for lymphocyte stimulation, partially purified virus, density gradient-purified virus, and soluble viral antigens were incubated with sensitized lymphocytes. The soluble viral antigens produced a 2-fold stimulation, whereas the partially purified virus and density gradient-purified virus produced 13- and 26-fold stimulation, respectively.

To compare the ability of infectious and noninfectious virus to stimulate lymphocytes, cells from immunized and unimmunized animals were incubated with infectious virus, UV-inactivated virus, or conditioned medium. The data in Table 2 show that infectious virus was about 50% less effective in stimulating lymphocytes than UV-inactivated virus. The depressive effect of infectious virus on lymphocytes from unimmunized animals was even more marked. These findings show that infectious virus can depress DNA synthesis; however, in the case of the sensitized lymphocytes,

TABLE 2. Inhibitory effect of infectious HSV on the stimulation of lymphocytes

Antigen*	Stimulation ratio	
	Cells from HSV-immunized animals	Cells from control animals
Infectious HSV	4.33	0.03
UV-inactivated HSV	9.44	0.45

* Lymphocytes were incubated with infectious virus or the identical virus preparation after UV-inactivation.

the degree of stimulation produced by viral antigens is apparently greater than the depressive effect produced by the infectious virus.

The course of development of the cellular immune response after immunization is illustrated in Fig. 3. Rabbits were immunized with HSV or cell homogenate by the combined intravenous and intradermal routes. At various times thereafter, spleen cell cultures were prepared and incubated with HSV antigens or conditioned medium. A 5.5-fold stimulation could be detected within 5 days after immunization, and an 11-fold stimulation was observed at 7 days. At 15 days, the stimulation ratio had decreased to 4, and the lymphocytes remained responsive (2- to 4-fold above controls) for as long as 120 days. In comparison to the intravenous and intradermal routes, immunization by the corneal route resulted in maximal stimulation (20- to 30-fold above controls) at 13-20 days after immunization, and at 40 days, the stimulation ratio was still 11-fold above controls.

Stimulation of sensitized lymphocytes by vaccinia virus

That lymphocyte stimulation can be used to study the cellular immune response to viruses other than HSV is illustrated in Table 3. Peripheral blood leukocytes, obtained from rabbits 2 weeks after immunization with vaccinia virus, were incubated with UV-inactivated vaccinia (equivalent to 4.0×10^6 PFU before UV-inactivation), cell homogenate, or conditioned medium. Peripheral leukocytes from unimmunized animals were used as controls. Vaccinia produced a 7-fold stimulation of leukocytes from immunized animals, but failed to stimulate leukocytes from unimmunized animals. In fact, vaccinia depressed the incorporation of [3 H]thymidine by control leukocytes (stimulation ratio 0.18). Preliminary experiments indicate that considerably less depression occurred with density gradient-purified vaccinia, suggesting

FIG. 3. Time of appearance of sensitized lymphocytes after immunization with HSV. (●—●), lymphocytes from immunized animals; (○—○), lymphocytes from control animals. Lymphocytes were incubated *in vitro* with HSV antigens or conditioned medium and the stimulation ratio was determined.

TABLE 3. *In vitro* stimulation of peripheral lymphocytes by vaccinia virus

Test material	Cells from vaccinia-immunized animals		Cells from unimmunized animals	
	[³ H]Thymidine uptake (cpm)	Stimulation ratio	[³ H]Thymidine uptake (cpm)	Stimulation ratio
Conditioned medium	4,740	—	4,218	—
Cell homogenate	2,983	0.63	4,245	1.00
Vaccinia virus	35,197	7.38	744	0.18

that a toxic factor or inhibitor might in part account for these results. Other experiments designed for determination of the factors and conditions required to produce maximal stimulation of lymphocytes *in vitro*, revealed that the stimulation ratio was highest at 72 hr after incubation with vaccinia, and that the degree of stimulation was directly related to the concentration of antigen. As in the case of HSV, virion-associated, rather than soluble, antigens were found to be responsible for the majority of the stimulation.

The time course of the cellular immune response to vaccinia is illustrated in Fig. 4. Rabbits were immunized with vaccinia, and at various intervals spleen cells or peripheral blood leukocytes were obtained and incubated with UV-inactivated virus or conditioned medium. Maximum stimulation occurred between 11 and 14 days after immunization and declined rapidly over the next several days. The fact that vaccinia stimulates peripheral leukocytes and that these cells can be obtained without sacrificing the animal suggests that *in vitro* stimulation of peripheral leukocytes might be a convenient technique for measurement of the cellular immune response to other viral infections.

Specificity of lymphocyte stimulation by viral antigens

To study the specificity of lymphocyte stimulation by viral antigens, spleen cells obtained from animals that had been immunized with HSV or vaccinia were incubated *in vitro* with viral antigens or conditioned medium. The data in Table 4 show that lymphocytes from HSV-immunized animals were stimulated only by HSV antigens, while lymphocytes from vaccinia-immunized animals were stimulated only by vaccinia antigens.

Effect of anti-HSV antibody on the stimulation of sensitized lymphocytes by HSV antigens

Finally, an experiment was designed to see whether incubation of viral antigens with anti-viral antibody affects lymphocyte

TABLE 4. *Specificity of stimulation of sensitized lymphocytes by viral antigens*

Antigen	Stimulation ratio	
	Cells from HSV-immunized animals	Cells from vaccinia-immunized animals
HSV	9.86	0.27
Vaccinia	0.80	5.48

FIG. 4. Time of appearance of sensitized lymphocytes in the spleen and peripheral blood after immunization with vaccinia. Lymphocytes were incubated *in vitro* with vaccinia antigens or conditioned medium and the stimulation ratio was determined.

stimulation. UV-inactivated HSV was incubated with serial 2-fold dilutions of rabbit anti-HSV serum or normal rabbit serum for 90 min at 37°. Portions from each reaction mixture then were added to sensitized lymphocytes and the degree of stimulation was determined. The ability of the anti-viral antibody to neutralize 10^{6.0} PFU of infectious virus was determined in a separate experiment. Fig. 5 shows that the antiserum against HSV had no inhibitory effect on the ability of HSV antigens to stimulate sensitized lymphocytes, despite the fact that at dilutions below 1/128 the antiserum was able to neutralize 100% of the virus.

DISCUSSION

Our studies with HSV and vaccinia point to several factors that appear to be particularly important in the stimulation of sensitized lymphocytes by viral antigens and should be taken into consideration when extending this technique to other viral infections. First, to reduce contaminating tissue culture antigens, to eliminate soluble factors in the crude virus pool that might be cytotoxic for lymphocytes, and to obtain sufficient quantities of viral antigens to produce

FIG. 5. Effect of different concentrations of anti-HSV antibody on the stimulation of sensitized lymphocytes by HSV antigens.

maximal stimulation, the crude virus should be concentrated and purified. Second, since certain viruses can replicate in macrophages and/or lymphocytes (14–17), and since macrophages play a facilitative role in the stimulation of lymphocytes (18, 19), infection of either of these cell types might depress the ability of viral antigens to stimulate lymphocytes. In fact, it has been observed that infectious virus can markedly depress the ability of lymphocytes to respond to phytohemagglutinin or nonviral antigens (20–22). Our studies with HSV suggest that infectious virus also can depress the lymphocyte response to viral (i.e., HSV) antigens. For this reason, noninfectious (UV-inactivated) virus should be used as the source of the viral antigen. Third, our studies suggest that maximal sensitization of lymphocytes may occur over a relatively short time range after immunization. Thus, the time at which lymphocytes are harvested after infection may be extremely important in detection of sensitized lymphocytes. Whether the short-lived nature of the peak lymphocyte response represents rapid elimination of viral antigens, virus-induced destruction of lymphocytes, a short-lived T-cell response, the response of some B-cells, or the influence of other factors remains to be determined.

The role of the cellular versus the humoral immune response in viral infections and the relationship between these two responses has been the subject of considerable speculation. The lymphocyte stimulation technique should make it possible to investigate these responses *in vitro*. Studies by other investigators with soluble, nonviral antigens indicate that specific antibody can enhance or inhibit the degree of lymphocyte transformation, depending on the ratio of antigen to antibody (23). In our system, concentrations of antibody that neutralized 100% of the infectious virus had no effect on the degree of lymphocyte stimulation. Although the failure of antiviral antibody to inhibit lymphocyte stimulation may be due to an excess of noninfectious viral antigens, dissociation of virus-antibody complexes, or the presence of antigenic sites to which the host had not formed antibody, other explanations must be considered. For example, macrophages may take up virus-antibody complexes and process the viral antigen for presentation directly or indirectly to lymphocytes. If the ability of HSV-anti-HSV complexes to stimulate sensitized lymphocytes can be confirmed with other viruses, this would suggest that the host has evolved a highly effective way of protecting the cells of the immune system from destruction by infectious virus, while at the same time allowing viral antigens to stimulate the immune system. Moreover, the ability of virus-antibody complexes to stimulate sensitized lymphocytes might prove to be a useful tool for detection of circulating noninfectious virus-antibody complexes in chronic viral infections.

There are several mechanisms by which cell-mediated immunity might defend the host against viral infections. Sensitized lymphocytes might react with and destroy virus-infected cells by processes similar to those involved in the cell-mediated destruction of tumors and grafts. Moreover, the release of lymphotoxin, migration inhibitory factor, chemotactic factors, interferon, and other biological mediators from stimulated lymphocytes might contribute, at least in part, to

the inhibition of viral replication and the destruction of virus-infected cells (1). Recent studies have shown that the humoral immune response of the host also can result in the destruction of virus-infected cells through the interaction of anti-viral antibody and complement with virus-induced cell surface antigens (24). Whether the attachment of sublytic concentrations of anti-viral antibody and complement to the surface of infected cells can accelerate the phagocytosis of these cells by activated macrophages, or can block the action of sensitized lymphocytes, remains to be investigated.

In conclusion, the *in vitro* lymphocyte stimulation technique should make it possible to assess the cellular immune response to viral antigens in various viral infections, to look for selective defects in cellular immunity to viral antigens in specific disease states, to study the release of biological mediators from sensitized lymphocytes after stimulation with viral antigens, and to study the relationship between the cellular and humoral immune responses in viral infections.

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