CELLULAR IMMUNITY TO HERPES SIMPLEX VIRUS MEDIATED BY INTERFERON

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Several lines of evidence suggest that cellular immunity plays an important role in the defense against herpes simplex virus (HSV)[1] infections. First, HSV can persist both in vivo and in vitro despite the presence of high concentrations of neutralizing antibody (1). Second, HSV infections are more severe in patients with deficiencies in cellular immunity (2–4). Third, experimental suppression of the cellular immune response makes animals more susceptible to HSV infection (2, 4). The precise way by which cell-mediated immunity operates to protect the host against HSV is far from clear; but recent studies suggest that different immunological mechanisms are required to control different modes of viral spread (5). For example, in the typical lytic infection, HSV can spread by either the extracellular route (Type I spread) or directly from cell-to-contiguous-cell (Type II spread) presumably as a result of cell fusion. Type I spread is readily stopped by antiviral antibody that neutralizes extracellular virus. Type II spread is not stopped by antiviral antibody because the intracellular location of the virus protects it from neutralization.

Little is still known about how the host combats Type II spread, but theoretically, the immune response could stop Type II spread by acting on one or more of three different sites. First, it could act by destroying virus-infected cells. Second, it could break cell-to-cell contact or prevent cell fusion; if this happens the virus would have to travel by the extracellular route to infect adjacent cells and the virus could then be neutralized by antiviral antibody. Third, if the host's immune response leads to the destruction of the surrounding uninfected cells or inhibits viral replication in these cells, the spread of virus would be halted.

Evidence that the immune response of the host can directly or indirectly act at each of these sites is beginning to accumulate. It is known that HSV can induce new antigens on the surface of infected cells and that antiviral antibody and complement can react

[1] *Abbreviations used in this paper:* CFA, complete Freund's adjuvant; GM, growth medium; HSV, herpes simplex virus; IFA, incomplete Freund's adjuvant; MEM, minimum essential medium; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; PFU, plaque-forming units; PPD, purified protein derivative; RK, rabbit kidney; VSV, vesicular stomatitis virus.

with these antigens and destroy the infected cell (6). The critical question, however, is not whether the infected cell is immunologically destroyed, but whether the destruction occurs before the virus is transmitted to contiguous infected cells. If the infected cell is immunologically destroyed after the virus is transmitted to contiguous uninfected cells, the infection would not be stopped. Recent in vitro studies suggest that this latter situation is what occurs in HSV infections (7). The failure to halt the spread of HSV by an immunological attack directed specifically at infected cells suggested that factors which could disrupt the connection between contiguous cells or could inhibit viral replication in adjacent cells might be needed to stop viral spread. Support for this contention comes from experiments which showed that when peritoneal exudate leukocytes, obtained from animals that had never been exposed to HSV, were added to monolayers of cells infected with HSV, the spread of the virus was inhibited (7). High concentrations of viable leukocytes were required, and they appeared to act by exerting a toxic effect on the underlying monolayer. This disrupted cell-to-cell contact and thereby allowed antiviral antibody and complement to destroy infected cells before cell-to-cell contact was reestablished. Supernatant fluids from these cultures did not inhibit the formation of HSV plaques nor contain measurable amounts of interferon. It was concluded that the leukocytes acted primarily by preventing cell-to-cell contact and thereby converting a Type II into a Type I spread.

The present experiments were initiated to see (*a*) whether leukocytes sensitized and stimulated by a specific antigen were more effective than unsensitized leukocytes in inhibiting viral replication and (*b*) whether soluble mediators released from sensitized and stimulated leukocytes could inhibit viral replication by acting on uninfected cells.

Materials and Methods

Tissue Culture and Media.—Rabbit kidney (RK) cells were prepared as previously described (8). Growth medium (GM) consisted of Eagle's minimum essential medium (MEM) with L-glutamine, 10% heat-inactivated fetal calf serum and antibiotics (100 U of penicillin-G/ml and 1.0 μg of fungizone/ml). After the RK cells had grown to confluency, they were removed by trypsinization, washed, resuspended in MEM with 20% calf serum and 10% dimethyl sulfoxide, and stored in liquid nitrogen. From this stock cell preparation, third and fourth passage RK monolayers were prepared and used. Dulbecco's phosphate-buffered saline (PBS) with Ca^{++} and Mg^{++} was used to wash monolayers.

Viruses.—HSV (type 1) was prepared and assayed in RK cells (8). To prevent development of secondary plaques, 2% pooled human serum containing antibody to HSV-1 was added to the growth medium (antibody overlay medium). Vesicular stomatitis virus (VSV), (Indiana strain), obtained from the American Type Cell Culture Collection, Rockville, Md. was grown and assayed in RK cells.

Antigens.—Tuberculin-purified protein derivative (PPD) was obtained from Parke, Davis and Co., Detroit, Mich. 100 ml of HSV-1, containing 1×10^8 plaque-forming units (PFU)/ml, were centrifuged at 81,000 *g* for 1 h. The virus pellet was suspended in 20 ml PBS, sonicated, and exposed for 10 min at 15 cm to two 15-watt germicidal lamps (General Electric Co., Schnectady, N.Y.). The UV-inactivated virus then was recentrifuged at 81,000 *g* for 1 h, suspended in 10 ml GM, sonicated, and stored at −70°C until used. All UV-inactivated HSV preparations were tested and found to be free of infectious virus.

Sensitization of Leukocytes.—New Zealand white rabbits, weighing 2–3 kg, were immunized subcutaneously with complete Freund's adjuvant (CFA) containing 5.0 mg/ml of *Mycobac-*

terium tuberculosis (strain Jamaica); each animal received 1 ml divided between the hind footpads and back of the neck. Rabbits immunized subcutaneously with incomplete Freund's adjuvant (IFA) served as controls. Skin tests were performed 1 wk before harvest of leukocytes with 5 μg of PPD. A test was considered positive if the redness and induration exceeded 20 × 20 mm at 48 h. Splenic leukocytes or peritoneal exudate cells (PEC) were harvested 3–6 mo after immunization.

Rabbits were immunized against HSV by intravenous inoculation of 3×10^8 PFU of infectious virus. Splenic leukocytes or PEC were harvested 9–12 days after immunization (9). Skin tests were not performed on HSV-inoculated rabbits.

Harvest of Sensitized Leukocytes.—Rabbit PEC were collected 3 days after the intraperitoneal injection of 50 ml of sterile 12.5% sodium caseinate (7). Approximately 70–80% of the PEC from both *M. tuberculosis*- and HSV-sensitized rabbits were macrophages or monocytes; the remainder were polymorphonuclear leukocytes and small lymphocytes. More than 92% of the cells were viable as measured by exclusion of trypan blue. Except where indicated otherwise, PEC will be referred to hereafter as sensitized leukocytes.

Spleens were obtained aseptically, trimmed of excess adipose tissue, minced with scissors, and suspended in GM by gently forcing the mince through a stainless steel screen. The suspended cells then were filtered through a double layer of sterile gauze and centrifuged at 800 *g* for 5 min. The pellet was exposed to 15 ml of 0.2% NaCl for 30 sec to lyse erythrocytes. Immediately thereafter, 15 ml of 1.6% NaCl was added; the cells then were centrifuged and resuspended in either GM or antibody overlay media. 80–85% of the spleen cells were viable as measured by exclusion of trypan blue.

Incubation of Sensitized Leukocytes with HSV-Infected Monolayers.—Confluent monolayers of RK cells in trays (Linbro Chemical Co., Inc., New Haven, Conn.) containing 24 wells (16 mm in diameter) were washed once with PBS and incubated at 37°C with approximately 30 PFU of HSV suspended in 0.2 ml of GM. At the end of 2 h the inocula were aspirated and the monolayers washed. Either CFA-sensitized leukocytes plus PPD in 2 ml of antibody overlay medium or HSV-sensitized leukocytes plus UV-inactivated HSV in 2 ml of GM (without antibody) were added to each well. In most instances, the leukocytes were used at a leukocyte-to-RK cell ratio of 10 (3×10^6 leukocytes/3×10^5 RK cells). At the end of 48 h the leukocytes and medium were aspirated and the monolayers were washed three times with PBS. The RK cells then were removed by scraping, resuspended in 1 ml of GM, frozen and thawed three times, and the titer of intracellular virus was determined (7). Titrations were done in triplicate, the data were averaged and expressed as PFU (log 10) per well. Most experiments were repeated three or more times.

Preparation of Supernatant Fluids from Sensitized Leukocytes.—Monolayers of uninfected RK cells were incubated with sensitized leukocytes obtained from animals immunized with either CFA or HSV. PPD or UV-inactivated HSV then was added and supernatant fluids were harvested at different times thereafter. In these experiments, anti-HSV antibody was not included in the overlay media. Supernatant fluids were centrifuged at 1,500 *g* for 30 min and stored at −70°C. The fluids were reconstituted with an equal vol of GM and then incubated with fresh uninfected monolayers. At appropriate intervals thereafter, the fluids were aspirated, the monolayers washed three times and challenged with approximately 30 PFU of HSV. 24 h after infection the monolayers were washed, the cells were removed and titrated for infectious virus (7).

Interferon Assay.—1 ml of supernatant fluids from CFA- or HSV-sensitized leukocytes was diluted with an equal vol of GM and incubated overnight with fresh uninfected RK monolayers. The supernatant fluids then were removed and the monolayers were challenged with 50 PFU of VSV. Interferon titers were expressed as the reciprocal of the highest dilution of the supernatant fluid that inhibited plaque formation by 50%.

RESULTS

Inhibition of HSV Replication by Leukocytes from Animals Immunized with CFA.—Monolayers infected with HSV were incubated with leukocytes from rabbits that had been immunized with either CFA or IFA. PPD was added, and 48 h later the virus titer was determined. The data in Table I show that in the absence of leukocytes, PPD did not inhibit viral replication. Similarly, PPD added to leukocytes from animals immunized with IFA failed to inhibit viral replication in excess of that produced by leukocytes alone (7). However, when PPD was added to leukocytes from animals immunized with CFA, the virus titer was reduced from $10^{4.2}$ to $10^{2.8}$ PFU.

Effect of CFA-Sensitized Leukocytes on Viral Replication at Different Times after Infection.—To determine how rapidly CFA-sensitized leukocytes exerted their inhibitory effect, monolayers that had been infected with HSV were incubated with sensitized leukocytes plus PPD, and at different times thereafter the virus titer was determined. The data in Table II show that within 24 h

TABLE I

*Inhibition of HSV Replication by Leukocytes from Rabbits Immunized with Incomplete or CFA**

Treatment	Leukocytes		
	Absent	Present	
		IFA	CFA
	PFU, log_{10}		
No PPD	5.5	4.3	4.2
PPD	5.5	4.3	2.8

* Monolayers infected with approximately 30 PFU of HSV were incubated with 5 μg of PPD plus leukocytes at a leukocyte-to-RK-cell ratio of 10. At the end of 48 h, the leukocytes were removed and the monolayers washed and titrated for virus.

TABLE II

*Effect of CFA-Sensitized Leukocytes on HSV Replication at Different Times After Infection**

H after infection	Leukocytes			
	Absent		Present	
	No PPD	PPD	No PPD	PPD
	PFU, log_{10}			
24	3.9	4.0	3.3	2.8
48	5.0	5.0	3.4	1.4
72	5.3	5.1	2.5	0

* Monolayers infected with approximately 30 PFU of HSV were incubated with 5 μg of PPD plus CFA-sensitized leukocytes at a leukocyte-to-RK-cell ratio of 10. At times indicated, the leukocytes were removed and the monolayers were washed and titrated for virus.

after infection, the virus titer in monolayers incubated with sensitized leukocytes plus PPD was $10^{2.8}$ PFU as compared to $10^{3.3}$ PFU in monolayers incubated with sensitized leukocytes but no PPD. At 48 h the virus titer was reduced by 99%; at 72 h no virus was detected. In contrast, monolayers that had been incubated with PPD, but without leukocytes, contained approximately $10^{5.0}$ PFU of HSV.

In other experiments (data not shown), CFA-sensitized leukocytes plus PPD were added to monolayers 24 h after initiation of the infection. 48 h later, the virus titer was reduced by >99% as compared to that in monolayers incubated with a similar concentration of leukocytes but without PPD.

Comparison of the Inhibitory Effect of CFA-Sensitized Leukocytes from the Peritoneal Cavity and Spleen.—Peritoneal and splenic leukocytes were obtained from the same rabbit and incubated with HSV-infected monolayers at a leukocyte-to-RK-cell ratio of 10. The data in Table III show that in the absence of PPD, splenic leukocytes did not inhibit viral replication, whereas peritoneal leukocytes produced the expected inhibition of viral replication (7). In the presence of PPD, peritoneal leukocytes completely inhibited viral replication, whereas splenic leukocytes reduced the virus titer from $10^{5.7}$ to $10^{3.8}$.

Effect of Different Concentrations of CFA-Sensitized Leukocytes on Viral Replication and Plaque Formation.—The data in Table IV show that in the absence of PPD, low concentrations of sensitized leukocytes did not inhibit viral replication. Only when the ratio of leukocytes to RK cells equalled or exceeded 2.5 was viral replication inhibited. In the presence of PPD, however, there was far greater inhibition of viral replication. The degree of inhibition was dependent upon both the concentration of leukocytes and the concentration of PPD. At a leukocyte-to-RK-cell ratio of 10, PPD inhibited viral replication by 3–4 logs more than that produced by leukocytes alone. Maximal inhibition was produced by 10 μg of PPD. Even more important is the fact that PPD inhibited

TABLE III

*Inhibition of HSV Replication by CFA-Sensitized Leukocytes from Spleen and Peritoneal Exudate**

Concentration of PPD	Leukocytes		
	Absent	Present	
		Spleen	Peritoneal exudate
μg		*PFU, log_{10}*	
0	5.7	5.7	4.7
10	5.8	4.3	0
100	6.1	3.8	0

* Monolayers infected with approximately 30 PFU of HSV were incubated with PPD plus leukocytes at a leukocyte-to-RK-cell ratio of 10. Peritoneal and splenic leukocytes were harvested from the same CFA-sensitized rabbit. At the end of 48 h, the leukocytes were removed and the monolayers washed and titrated for virus.

TABLE IV

*Inhibition of HSV Replication by Different Concentrations of CFA-Sensitized Leukocytes**

Ratio of leukocytes to RK cells	Concentration of PPD (μg)			
	None	1	10	100
	PFU, log_{10}			
No leukocytes	6.2	6.3	6.2	6.3
0.1	6.3	6.1	5.0	4.8
0.25	6.3	5.4	4.2	4.6
0.5	6.0	5.2	4.2	4.0
1.0	6.2	4.8	3.2	3.5
2.5	5.7	3.0	3.2	3.2
5.0	4.7	2.9	2.0	2.2
10.0	4.2	0.9	0.3	0.9

* Monolayers infected with approximately 30 PFU of HSV were incubated with different concentrations of CFA-sensitized leukocytes and PPD. At the end of 48 h the leukocytes were removed and the monolayers were washed and titrated for virus.

viral replication by over 90% when the ratio of CFA-sensitized leukocytes to RK cells was as low as 0.1.

To show that the inhibition of viral replication by leukocytes in the absence of PPD was not due to an in vitro allograph reaction, RK cells and peritoneal leukocytes were obtained from the same rabbit. In each of three separate experiments (data not shown), HSV replication was inhibited to about the same degree as noted in column 2 of Table IV.

The effect of different concentrations of CFA-sensitized leukocytes on the formation of plaques is illustrated in Table V. In the absence of PPD, low concentrations of leukocytes did not inhibit plaque formation. Only when the ratio of leukocytes to RK cells was 2.5 or greater was plaque formation inhibited by 50%. In the presence of PPD, plaque formation was markedly inhibited by a leukocyte-to-RK cell ratio of 0.5. In general, the plaques that did develop after exposure to PPD-stimulated leukocytes were considerably smaller than those that appeared after exposure to unstimulated leukocytes.

Inhibition of HSV Replication by Supernatant Fluids from CFA-Sensitized Leukocytes.—Previously we found that high concentrations of unsensitized leukocytes were toxic to monolayers (7), and that this toxicity was at least in part responsible for preventing the cell-to-cell spread of HSV infections. The data in Tables IV and V of the present report show that low concentrations of PPD-stimulated leukocytes (ratio $\leq$ 0.5) also can inhibit the spread of HSV. Microscopic examination of monolayers exposed to low concentrations of stimulated leukocytes showed that stimulated leukocytes adhered more firmly to monolayers than unstimulated leukocytes but revealed little, if any, evidence of cytotoxicity. Studies measuring the release of ^{51}Cr from monolayers treated with low concentrations of PPD-stimulated leukocytes also failed to provide

TABLE V

*Inhibition of Plaque Formation by Different Concentrations of CFA-Sensitized Leukocytes**

Ratio of leukocytes to RK cells	PPD	
	Absent	Present
	No. of plaques	
No leukocytes	29	27
0.10	23	23
0.25	23	17
0.50	21	5
1.0	17	4
2.5	9	0
5.0	6	0
10.0	3	0

* Monolayers infected with approximately 30 PFU of HSV were incubated with different concentrations of CFA-sensitized leukocytes, plus 100 μg of PPD. At the end of 48 h the leukocytes were removed, the monolayers were washed, and the number of plaques counted.

evidence for cell damage (data not shown). The fact that low concentrations of CFA-sensitized leukocytes exposed to PPD inhibited viral replication without producing cytotoxicity, while high concentrations of unstimulated leukocytes inhibited viral replication as a result of cytotoxicity, suggested that different mechanisms were involved in these two situations. For this reason we began to suspect that an immunologically induced mediator was being produced by the PPD-stimulated leukocytes.

To test this possibility, CFA-sensitized leukocytes plus PPD were incubated with uninfected RK cells for 24 h. Supernatant fluids were harvested from these cultures and mixed with an equal vol of GM. The reconstituted supernatant fluids were incubated with fresh uninfected monolayers for 3, 12, 24, or 48 h. The monolayers then were washed and challenged with HSV. The data in Table VI show that supernatant fluids markedly inhibited viral replication when added to monolayers for 12 or more h before challenge with HSV. Viral replication was not substantially inhibited if the monolayers were exposed to the supernatant fluids for only 3 h before challenge. Supernatant fluids from CFA-sensitized leukocyte cultures that had not been exposed to PPD failed to protect the monolayers against HSV. Supernatant fluids of leukocytes from animals injected with IFA and incubated with PPD also failed to inhibit viral replication (data not shown).

Comparison of the viral inhibitory activity of supernatant fluids from CFA-sensitized peritoneal leukocyte cultures with CFA-sensitized splenic leukocyte cultures revealed marked inhibitory activity in the former within 2 h after exposure to PPD while relatively little activity was found in the latter despite exposure to PPD for as long as 24 h (data not shown).

Number of CFA-Sensitized Leukocytes Required to Produce Inhibitor in Super-

TABLE VI

*Inhibition of HSV Replication by Supernatant Fluids from CFA-Sensitized Leukocytes Incubated with PPD**

Concentration of PPD used to prepare supernatant fluids	Time (h) that monolayers were exposed to supernatant fluids before challenge with HSV			
	3	12	24	48
μg	*PFU, log_{10}*			
GM control‡	4.8	3.9	4.5	3.9
0	4.8	4.0	4.1	3.7
10	4.4	1.5	1.0	1.0
100	4.4	0	0	0

*CFA-sensitized leukocytes plus PPD were incubated for 24 h with uninfected RK cells at a leukocyte-to-RK-cell ratio of 10. The supernatant fluids were removed and diluted with an equal vol of GM. Uninfected monolayers then were exposed to either the reconstituted supernatant fluids or fresh GM. At various times thereafter, the monolayers were washed and challenged with approximately 30 PFU of HSV. 24 h after challenge the monolayers were washed and the cells titrated for virus.

‡ Fresh GM (no leukocytes or PPD).

natant Fluid.—Different concentrations of CFA-sensitized leukocytes plus PPD were incubated with uninfected RK cells. 24 h later the supernatant fluids were harvested and reconstituted with an equal vol of GM. Fresh uninfected RK monolayers were incubated with the reconstituted supernatant fluids for 24 h, washed, and then challenged with HSV. The data in Table VII show that supernatant fluids obtained from RK cultures exposed to leukocytes at a ratio of 0.005 reduced the virus titer from $10^{3.6}$ to $10^{2.4}$. Thus, under the conditions of our system, approximately 1,500 leukocytes or less than one leukocyte per 200 RK cells inhibited HSV replication by over 90%.

Properties of Inhibitor in Supernatant Fluid.—The inhibitor was nondialyzable, did not sediment at 100,000 *g* for 1 h, was relatively resistant to treatment at pH 2.0 for 24 h at 4°C (titer reduced from 1:256 to 1:128), lost some activity when heated at 56°C for 1 h (titer reduced from 1:256 to 1:64), and was inactivated by exposure to trypsin (1.25 mg/ml) for 5 h at 37°C. The inhibitor did not neutralize HSV, it was species specific in that it did not inhibit replication of VSV or HSV in mouse L cells, but it was not virus specific in that it inhibited HSV and VSV in RK cells. From these studies we conclude that the inhibitor is interferon. When compared with a rabbit interferon standard obtained from the National Institute of Allergy and Infectious Diseases, the supernatant fluid prepared from 3×10^6 CFA-sensitized leukocytes that had been incubated with 100 μg of PPD for 24 h, contained at least 256 U of interferon/ml.

Inhibition of HSV Replication by HSV-Sensitized Leukocytes.—The experiments described above showed that CFA-sensitized leukocytes stimulated by an antigen (PPD) unrelated to HSV could inhibit HSV replication. The next experiments were performed to see if leukocytes specifically sensitized to HSV

TABLE VII

*Inhibition of HSV Replication by Supernatant Fluids Produced with Different Concentrations of CFA-Sensitized Leukocytes**

Ratio of leukocytes to RK cells used to prepare supernatant fluids	Virus titer
	PFU, log_{10}
PPD control‡	3.9
GM control§	3.6
0.005	2.4
0.01	2.5
0.025	2.3
0.05	1.7
0.1	0
0.25	0

* Different concentrations of CFA-sensitized leukocytes plus 100 μg of PPD were incubated for 24 h with uninfected RK cells. The supernatant fluids were removed, diluted with an equal vol of GM, and incubated with uninfected RK monolayers for 24 h. The monolayers then were washed, challenged with approximately 30 PFU of HSV, and 24 h later titrated for virus.

‡ No leukocytes.

§ Fresh GM (no leukocytes or PPD).

also could inhibit HSV replication. Peritoneal and splenic leukocytes obtained from HSV-immunized rabbits were added to monolayers infected with approximately 30 PFU of HSV; UV-inactivated viral antigen (1.0×10^6 to 1.0×10^8 PFU) then was added to stimulate the sensitized leukocytes. The data in Table VIII show that in the absence of UV-inactivated virus, peritoneal leukocytes produced the usual reduction in virus titer. However, when UV-inactivated virus was added, HSV replication was inhibited to a much greater extent (e.g., at a peritoneal leukocyte-to-RK-cell ratio of 10, the virus titer was reduced by over 99.9% from $10^{5.9}$ to $10^{2.7}$ PFU). In the absence of leukocytes, UV-inactivated virus added to RK cells did not inhibit HSV replication. In general, UV-inactivated virus incubated with unsensitized leukocytes produced relatively little inhibition of viral replication, although in some experiments between 30% and 90% inhibition was observed (data not shown). As in the case of CFA-sensitized leukocytes, the degree of viral inhibition produced by HSV-sensitized leukocytes was greater with peritoneal than with the splenic leukocytes and was dependent upon both the concentration of leukocytes and the concentration of antigens.

Inhibition of HSV Replication by Supernatant Fluids from HSV-Sensitized Leukocytes.—The data in Table IX show that supernantant fluid from HSV-sensitized leukocytes that had been exposed to UV-inactivated virus completely inhibited HSV replication. In contrast, supernatant fluid from HSV-sensitized leukocytes that had not been exposed to UV-inactivated virus produced substantially less inhibition of viral replication. Characterization of the inhibitor

TABLE VIII

*Inhibition of HSV Replication by HSV-Sensitized Leukocytes**

UV-inactivated HSV‡	Ratio of leukocytes to RK cells§				
	None	Splenic		Peritoneal	
		5:1	10:1	5:1	10:1
		PFU, log_{10}			
None	7.9	7.6	7.4	6.4	5.9
1×10^6	8.0	7.5	7.2	6.4	4.8
1×10^8	7.7	6.9	6.0	3.7	2.7

* Monolayers infected with approximately 30 PFU of HSV were incubated with HSV-sensitized splenic or peritoneal leukocytes plus different concentrations of UV-inactivated HSV. Anti-HSV antibody was not included in the overlay media. At the end of 48 h the monolayers were washed and titrated for infectious virus.

‡ Number of PFU before UV-inactivation.

§ Leukocytes were harvested 9 days after intravenous immunization with 3×10^8 PFU of infectious HSV.

TABLE IX

*Inhibition of HSV Replication by Supernatant Fluids from HSV-Sensitized Leukocytes**

Preparation of supernatant fluid		Time that monolayers were exposed to supernatant fluids before challenge with HSV	
HSV-sensitized leukocytes†	Treatment‡	24 h	48 h
		PFU, log_{10}	
Absent	None§	4.6	4.6
Present	None§	3.7	3.5
Present	UV-inactivated HSV	0	0

* HSV-sensitized leukocytes, at a leukocyte-to-RK-cell ratio of 10, were incubated with 1×10^8 PFU of UV-inactivated HSV. Anti-HSV antibody was not included in the overlay media. 24 h later, the supernatant fluids were removed and reconstituted with an equal vol of fresh GM. The reconstituted supernatant fluids were then incubated with fresh uninfected RK monolayers for 24 or 48 h before challenge with approximately 30 PFU of HSV. 24 h after challenge, the monolayers were washed and titrated for virus.

‡ Leukocytes were harvested 12 days after intravenous immunization with 3×10^8 PFU of infectious HSV.

§ Fresh GM.

showed that it had the same properties as the interferon produced by PPD-stimulated leukocytes.

DISCUSSION

Previously we showed that high concentrations of unsensitized leukocytes could inhibit HSV replication and the development of viral plaques (7). The present study showed that sensitized leukocytes were even more effective in

inhibiting viral replication and plaque formation. The mechanisms by which unsensitized and sensitized leukocytes inhibit viral replication appear to be quite different. Unsensitized leukocytes (at high concentrations) acted by exerting a generalized cytotoxic effect. PPD-stimulated leukocytes (at low concentrations) were not cytotoxic but inhibited viral replication by producing interferon. In both situations, the inhibition was nonspecific (i.e., an HSV antigen recognition phase was not required). In the case of leukocytes sensitized to HSV, a specific HSV antigen recognition was required, but only to stimulate the leukocytes; the effector phase (i.e., interferon production) was again nonspecific.

Our studies also indicate that the mechanism of action of immunologically stimulated leukocytes (i.e., interferon production) is not only different from that of unsensitized leukocytes (i.e., cytotoxicity), but that the number of leukocytes required to inhibit viral replication is markedly different in the two situations. To inhibit viral replication with unsensitized leukocytes, a relatively high ratio of leukocytes to RK cells ($>2.5:1$) was required. In contrast, viral replication was inhibited by immunologically stimulated leukocytes at a ratio of leukocytes to RK cells of less than 0.1. When supernatant fluids from cultures of stimulated leukocytes were used, less than one leukocyte per 200 RK cells was capable of inhibiting viral replication by over 90%. This is considerably less cells than the number of immune leukocytes generally required to destroy specific target cells (10), and suggests that a relatively small number of leukocytes at the site of the lesion may be capable of stopping viral spread by reducing the susceptibility of the surrounding tissue.

Experiments from other laboratories also have shown that leukocytes from animals immune to a specific antigen (viral or nonviral) produced more interferon when exposed to that antigen than leukocytes from nonimmune animals (11–19). Recently, Youngner and Salvin (17) reported that immunologically induced interferon differs in its physical properties from nonimmunologically induced interferon; at pH 2.0 the interferon was inactivated. In our studies, however, the immunologically induced interferon was stable at pH 2.0. It is quite possible that different types and amounts of interferon may be produced by different subpopulations of leukocytes (21–23). This might be one of the explanations for the lower amount of interferon produced in our experiments by splenic as compared to peritoneal leukocytes. The specific cell type(s) producing interferon in our system also remains to be determined, but evidence from other laboratories indicates that both immune lymphocytes and macrophages can produce interferon (12, 15, 18).

Endotoxin is a common contaminant of biological materials and can lead to the release of interferon from leukocytes (24). The demonstration that PPD did not release interferon following incubation with either unsensitized or IFA-sensitized leukocytes argues against endotoxin contamination as a major factor in our experiments. Supernatant fluids from unsensitized leukocytes (7) and

CFA-sensitized leukocytes that had not been exposed to PPD also contained little, if any, interferon (Table VI). In contrast, supernatant fluids from HSV-sensitized leukocytes that had not been exposed to UV-inactivated virus contained some interferon (Table IX). Why supernatant fluids from unstimulated HSV-sensitized, but not unstimulated CFA-sensitized leukocytes contained interferon is not clear, but the explanation may lie in the fact that the CFA-sensitized leukocytes were harvested 3–6 months after immunization, whereas HSV-sensitized leukocytes were harvested 9–12 days after immunization. Conceivably HSV-sensitized leukocytes were still in an "activated" state from in vivo exposure to the live virus. Whether HSV-sensitized leukocytes obtained weeks or months after immunization also "spontaneously" release interferon has not yet been determined.

Another issue that remains to be resolved is whether immunologically induced interferon is really needed to stop the spread of HSV, since most of the noninflammatory cells in which HSV replicates (e.g., RK cells) are potentially capable of producing interferon. It is known, however, that certain viruses are poor inducers of interferon (20, 25). In our experiments little if any interferon was found in the supernatant fluids of RK cells exposed to either infectious or UV-inactivated virus (data not shown). In contrast, interferon was detected within several hours after stimulation of sensitized leukocytes. This suggests that interferon produced by leukocytes at the site of infection may be needed by the host to cope with viruses that otherwise are poor inducers of interferon. Moreover, immune leukocytes, in contrast to other target cells (e.g., fibroblast), can make interferon when exposed to antigens that do not contain nucleic acid (e.g., PPD). Theoretically, at least, this would enable the immune host to produce interferon in response to a variety of viral components (e.g., structural and nonstructural proteins) to which the nonimmune host would not react. Thus, cellular immunity may amplify the host's interferon-producing capacity.

In conclusion, the present study with CFA- and HSV-sensitized leukocytes supports and expands the concept that the host's immunological defense against HSV consists of two phases: an immunologically specific antigen recognition phase and a nonspecific effector phase (7) (Fig. 1). In the first phase, antiviral antibody, complement and immune leukocytes react with virus or virus-infected cells. This generates a variety of mediators (e.g., C5a, lymphocyte-derived chemotactic factor, migration inhibitory factor) which can attract and keep inflammatory cells at the site of the infection (26, 27). In addition, a number of other mediators including lymphotoxin and interferon are generated. The nonspecific phase of the defense consists of the action of these inflammatory cells and mediators on both infected and adjacent uninfected cells, thereby breaking cell-to-cell contact and inhibiting viral replication. Because the virus spreads so rapidly from cell-to-contiguous cells, the specific phase of the defense which destroys virus-infected cells appears to have little effect on halting the infection (7). It appears that it is the nonspecific phase of the defense, that is,

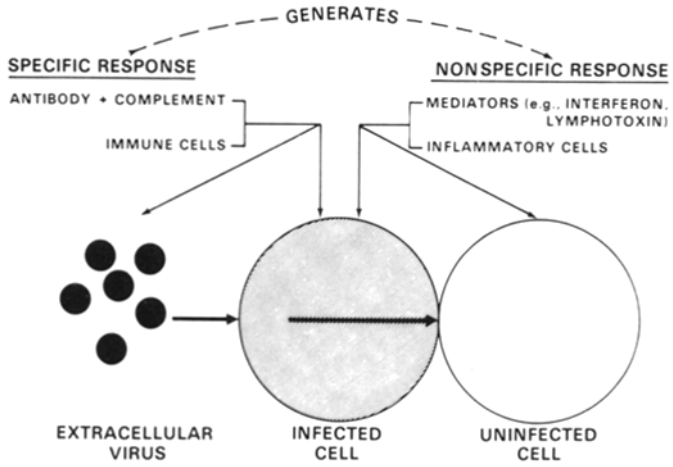

FIG. 1. Sites of action of the specific and nonspecific immune responses to HSV infection.

the nonspecific cytotoxicity produced by inflammatory cells and lymphotoxin and the inhibition of viral replication produced by immunologically-induced interferon, which is required to finally stop the cell-to-cell spread of HSV.

SUMMARY

Rabbit kidney cell monolayers infected with herpes simplex virus (HSV) were incubated with leukocytes from rabbits immunized with complete Freund's adjuvant. When the leukocytes were exposed to tuberculin purified protein derivative (PPD), viral replication and plaque formation were markedly inhibited. Similarly, when leukocytes from animals immunized with HSV were exposed to UV-inactivated HSV, viral replication was markedly inhibited. Exposure of leukocytes from unimmunized animals or animals immunized with incomplete Freund's adjuvant to UV-inactivated virus or PPD produced relatively little inhibition of viral replication. Examination of supernatant fluids from stimulated cultures revealed a soluble mediator that had the properties of interferon. Interferon production was detected within several hours after exposure of sensitized leukocytes to antigen. Supernatant fluids from as few as one sensitized leukocyte per 200 rabbit kidney cells inhibited HSV replication by over 90%. These findings support the concept that the cellular immune response to HSV consists of two phases: an immunologically specific antigen recognition phase, and a nonspecific effector phase that stops HSV spread by generating interferon.

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