Analysis of the Inhibitory Effect of Peritoneal Macrophages on the Spread of Herpes Simplex Virus

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Peritoneal macrophages obtained from mice after an intraperitoneal injection of tryptose peptone inhibited the development of herpes simplex virus type 2 plaques in syngeneic mouse embryonic fibroblasts. In contrast, peritoneal macrophages, spleen cells, and thymocytes from untreated mice showed only a minimal inhibitory effect on the development of viral plaques. The effect was age dependent. Macrophages from 2- and 3-week-old mice showed weaker functions, requiring a larger number of cells for an equivalent reduction of plaques and virus yield than those from adult mice. When macrophages were treated with procaine, their phagocytic activity was completely abolished. However the procaine-treated macrophages still could inhibit the development of viral plaques. Peritoneal macrophages did not show any increased cytotoxicity against herpes simplex virus-infected cells; plaque inhibition might rather be attributable to their cytostatic effects on target cells.

Intraperitoneal inoculation of newborn mice with herpes simplex virus (HSV) results in a rapid spread of virus to the viscera, followed by encephalitis and death, whereas similar inoculation of adult mice results in no viral spread or fatal disease (1, 9, 12). Johnson suggested that macrophage maturation might be responsible for the age-dependent resistance of mice on the ground that virus spread was seen both in vitro and in vivo with macrophages from suckling mice but not with those from adult mice (9). Additional evidence was that peritoneal macrophages transferred from adult mice could protect suckling mice against subsequent intraperitoneal infection with the virus (8). The mechanisms of protection seemed to be related to increased interferon production as well as to an increased ability to destroy the virus (8).

As previously reported by Lodmell et al. (14), HSV can spread by a cell-to-cell mode even under a high concentration of antibody and complement (5). Since the virus spreads rapidly from a cell to neighboring cells without being exposed to the fluid phase, the reaction of specific antibody and complement appears to have little ability to stop this spread of infection (4, 12, 14). How the host can combat this virus spread is not clear at the present time, but Lodmell et al. showed that a high concentration of macrophage-rich peritoneal leukocytes could prevent the development of HSV by yet unknown mechanisms (14).

We initiated the present study to see what

kind of cell in peritoneal exudate was actually responsible for the inhibition of viral plaques. We further wanted to answer the following questions: (i) whether antibody is required for these cells to prevent viral plaques, (ii) whether this function is also age dependent, (iii) whether the phagocytic activity of macrophages is needed for the plaque-preventing effect, and (iv) whether this inhibitory effect of macrophages is due to a cytotoxic effect or rather to a cytostatic effect on target cells.

MATERIALS AND METHODS

Mice. Inbred strain DDD mice were supplied by the Experimental Animal Center of our Institute and were bred in our laboratory.

Target cells and media. DDD mouse embryos were decapitated and dissected with scissors. A suspension of mouse embryo fibroblasts (MEF) was made by trypsinization, and approximately 5×10^5 cells per well were seeded in Linbro plastic tissue culture trays (16 mm in diameter) carrying 24 wells (Linbro Scientific Co., Inc., New Haven, Conn.). Growth medium (GM) consisted of Eagle minimal essential medium supplemented with 10% heat-inactivated calf serum and antibiotics (100 U of penicillin, 10 μ g of streptomycin, and 10 μ g of kanamycin per ml). Dulbecco's phosphate-buffered saline (PBS) was used to wash monolayers.

Virus. Seeds of HSV type 2 (HSV-2, UW 268 strain, 2×10^7 plaque-forming units [PFU]/ml) were prepared in primary rabbit kidney (PRK) cells or Vero cells. The Linbro tissue culture tray with 24 wells was used for assay of infectivity based on the average number of plaques per well. Cell-free HSV-2 containing 2×10^7

PFU/ml was inactivated by exposure to ultraviolet (UV) light for 15 min at a distance of 15 cm with constant swirling of the virus solution to assure even exposure.

Antisera. Antiserum against HSV-2 was prepared by bilateral corneal inoculation of New Zealand white rabbits. The neutralizing antibody titer exceeded 1:2,000. Anti-theta antibody was supplied by Michio Fujiwara, Department of Immunology of our Institute. This antibody was obtained by immunizing a rabbit with C3H mouse brain (2, 7), and its potency against theta-bearing T cells was confirmed in our previous experiments (17), in which it could effectively inhibit effector T-cell activity against vaccinia-infected target cells. Anti-sheep erythrocyte rabbit serum (hemolysin) was purchased from Microbiological Associates, Bethesda, Md.

Complement. Pooled normal rabbit sera were used as the source of complement.

Macrophages. Mice were injected intraperitoneally with 1 ml of sterile 2% tryptose peptone (Difco Laboratories, Detroit, Mich.). Three days later, the peritoneal cavity was exposed by midline incision and washed with PBS containing 0.02% ethylenediaminetetraacetate and antibiotics. The cells in the exudate were centrifuged at $800 \times g$ for 10 min and washed with PBS; then the erythrocytes were lysed by osmotic shock. The cells were centrifuged, resuspended in GM, and seeded in large glass bottles. After 12 to 16 h of incubation at 37°C, the media were discarded, and the cells were washed several times with PBS. Glass-adherent cells were removed with 0.02% ethylenediaminetetraacetate and 0.1% trypsin accompanied by gentle scrapings with a rubber policeman and were used as the source of macrophages. Over 90% of the cells were viable as determined by exclusion of trypan blue. These cells were stained with Giemsa and endogenous peroxidase, and, morphologically, over 95% of the cells were identified as macrophages. As a control, the peritoneal cavity of uninjected mice was opened, washed, and treated in the same manner as above, to obtain normal macrophages. Again, approximately 95% of these cells were identified as macrophages by Giemsa and endogenous peroxidase staining.

Incubation of macrophages with infected cells. Confluent monolayers of DDD MEF in tissue culture trays were washed once with PBS, and each well was infected with 20 to 25 PFU of HSV-2 suspended in 0.3 ml of GM. After incubation at 37°C for 2 h in a CO₂-air incubator, the inocula were removed, and the monolayers were washed once and then incubated with either 2 ml of overlay medium (GM) or overlay medium containing macrophages at macrophage to MEF ratios of 20:1, 10:1, 5:1, and 1:1. The monolayers were incubated at 37°C for 24 to 48 h; then the media were removed, and the monolayers were washed with PBS, fixed with methanol for a few minutes, and stained with Giemsa solution for counting of the number of plaques. For infectious virus titration, monolayers were disrupted by repeated freezing and thawing after 24 to 48 h of incubation; they were centrifuged to remove cell debris, and the supernatant was examined for virus in Vero cell cultures by a plaque method. All determinations were done in triplicate, and most experiments were repeated at least three times.

Effect of anesthetics on the phagocytic activity of macrophages. Lidocaine-HCl was kindly provided by Sankyo Pharmaceutical Co., Tokyo, Japan. Procaine-HCl was purchased from Daiichi Pharmaceutical Co., Tokyo, Japan. Macrophages were obtained from the peritoneal cavity of peptone-stimulated mice and were maintained in culture for 24 h on Lab-Tek two-chamber slides (Lab-Tek Products, Naperville, Ill.).

One milliliter of a 2% suspension of either hemolysin-coated or uncoated sheep erythrocytes, colloidal carbon (Pelikan carbon ink C11/1431a; Günther Wagner, Hannover, West Germany; 0.01 mg/ml), and 0.1 ml of polystyrene latex particles ($\rho = 0.109$, 0.2% solution) were mixed with GM containing anesthetics at final concentrations of 0.05 to 0.2%. Macrophages were exposed to these mixtures for 1 h at 37°C in an atmosphere containing 95% air and 5% CO₂. Then they were washed, stained with Giemsa solution, and checked for phagocytic activity under light and phase microscopes.

Effect of anesthetics on the inhibitory effect of macrophages. In a test designed to check whether macrophages treated with anesthetics were able to exert the inhibitory effect on the development of viral plaques, macrophages were suspended in GM containing an anesthetic at a concentration to make the macrophage to target cell ratio 20:1 and then added to MEF monolayers which had been infected with HSV-2 (UW 268 strain) 2 h earlier. Controls were incubated with macrophages alone or without either drug or macrophages. The monolayers which had been overlaid with macrophages suspended in GM containing an anesthetic were further incubated for 22 h at 37°C in a 5% CO₂ incubator; then the monolayers were washed once with PBS and fixed with methanol, and the viral plaques were counted.

⁵¹Cr labeling of target cells. Secondary MEF were grown on a Micro Test II tissue culture plate (Falcon Plastics, Oxnard, Calif.) carrying 96 wells. After labeling of MEF with 1 μ Ci of ⁵¹Cr per well for 2 h, the cells were infected with HSV-2 at a multiplicity of infection (MOI) of 5. Residual virus was removed after 2 h of adsorption, and macrophages suspended in GM or GM containing 0.1% procaine were then added onto the MEF and allowed to react with virus-infected monolayers for 16 h. ⁵¹Cr released in the media was assayed with a gamma counter, and specific lysis was calculated by the conventional method.

Assay of [¹²⁵I]IUDR incorporation. MEF target cells in various concentrations were plated in GM on Micro Test II tissue culture plates carrying 96 wells 24 h before the addition of macrophages. Virus-infected target cells were provided as follows. The medium was removed from each well and replaced with 0.1 ml of GM containing HSV-2 (MOI, 1.0 or 0.1) or UV-inactivated HSV-2 which had the same infective titer as the live virus before UV inactivation. After 2 h of exposure to the viruses, the wells were washed once with PBS, and the cells were used as target. The medium was aspirated, and macrophages at several ratios of macrophage to target cells were added and incubated together for 18 h at 37°C. The medium was then removed and replaced with 0.2 ml of medium containing 1.0 μ Ci of [¹²⁵I]IUDR (5-[¹²⁵I]-iodo-2'-

deoxy-uridine; Amersham Radiochemical Centre, Amersham, England). After a further 6 h of incubation at 37°C, the experiment was terminated by washing the trays in PBS. The trays were dried overnight, and individual wells were cut out with a power saw and the samples were counted in a gamma counter. Each experimental group consisted of six wells, and experiments were carried out twice.

Immunofluorescence. Anti-*HSV-2* immunoglobulin G from rabbit antiserum was purified by ammonium sulfate precipitation and labeled with fluorescein isothiocyanate by our standard method (10). Nonspecific fluorescence was eliminated by absorbing the conjugate with acetone-treated powder of mouse liver and kidney. Infected and uninfected MEF grown on cover glasses were fixed with acetone for 5 min at room temperature and then stained at 4°C overnight. After repeated washing with PBS, the cover glasses were mounted in buffered glycerol (pH 9.5) and observed under a fluorescence microscope.

Age-dependent mortality of infected mice. Mice of various ages were infected by the intraperitoneal route with 5×10^3 PFU of the UW 268 strain and observed every day for 2 weeks. The mortality rate was calculated from results obtained with at least 30 animals per group.

RESULTS

Inhibition of HSV-plaque formation by macrophages. For the study of the effect of macrophages on the development of HSV plaques, monolayers which had been infected with *HSV-2* (UW 268 strain) 2 h earlier were incubated with different concentrations of macrophages. At 48 h, the plaques were counted. As summarized in Table 1, the macrophages inhibited the development of viral plaques, an

TABLE 1. *Inhibition of the development of HSV plaques by activated macrophages*

Ratio of MEF to macrophages	Activated macrophages ^a		Normal macrophages ^b	
	No. of plaques ^c	Virus titer (PFU/ml) ^d	No. of plaques ^c	Virus titer (PFU/ml) ^d
1:1	18 ± 5.8	2.4×10^4	19 ± 0.7	2.5×10^4
1:10	9 ± 4.6 (<0.2)	2.8×10^3	16 ± 0.7	1.2×10^4
1:20	0.5 ± 1.0 (<0.001)	2.0×10^2	14 ± 2.0 (<0.2)	5.0×10^3

^a Activated peritoneal macrophages were obtained from adult DDD mice 4 days after intraperitoneal inoculation of tryptose peptone. Peritoneal macrophages were purified by glass adherence.

^b Peritoneal cavities of normal adult DDD mice were washed with PBS, and peritoneal macrophages were obtained in the same way as activated macrophages.

^c Mean number of plaques ± the standard error of the mean for 10 wells. Without macrophages, the number of plaques was 19 ± 3.7 . Statistical differences were evaluated between groups by Student's *t* test, and *P* values are shown in parentheses.

^d Virus titer in each well was assayed on Vero cells. Without macrophages, the virus titer was 2.8×10^4 PFU/ml.

almost complete suppression being observed at a macrophage-to-MEF ratio of 20:1. The plaque-inhibiting effect exerted by macrophages was proportional to the macrophage concentration. The amount of virus in the monolayer at 48 h postinfection was reduced by addition of macrophages, depending upon the concentration of macrophages. As a control, peritoneal macrophages were collected from normal DDD mice by simple washing of their peritoneal cavity with PBS. Infected monolayers incubated with a high concentration of the normal unstimulated macrophages also showed a considerable degree of inhibition of the development of viral plaques, but this inhibitory effect was far less than that of the macrophages induced by peptone. This conclusion was further confirmed by an immunofluorescence study. Monolayers were infected with *HSV-2* and at different times thereafter were examined for appearance of viral antigens. The number of foci and intensity of specific fluorescence in infected foci were markedly reduced by overlaying with peptone-induced macrophages.

Effect of anti-theta antibody plus complement on the plaque-inhibiting effect of macrophages. In the preceding experiments, the cell suspension used had been purified by glass adherence and was estimated on the basis of morphology to contain over 95% macrophages. Still, however, some lymphocytes existed in the preparation. To determine more precisely the type of cells which contributed to this inhibition and to eliminate the action of lymphocytes, a macrophage suspension was mixed with potent anti-theta antibody plus complement for 2 h and then was washed and added to monolayers that had been infected with *HSV-2*. Forty-eight hours later, plaques were counted. This treatment did not alter the inhibitory activity of the macrophage preparation. Thus, we concluded that the peptone-induced peritoneal macrophage did represent the cell which halted the development of viral plaques.

Comparison of the inhibitory effects of spleen cells, thymocytes, and macrophages. Whether the inhibition of viral plaques is a characteristic of the peritoneal macrophages or whether other immunocompetent cells can also exert this effect was determined. Thymocytes and spleen cells obtained from normal mice did not significantly inhibit the development of viral plaques, whereas peptone-induced macrophages produced the expected inhibition. At a high concentration (20:1), 80% of plaques were suppressed by peptone-induced macrophages, whereas thymocytes, spleen cells, and normal macrophages reduced the number of viral plaques only 20 to 25% (Table 1).

Effect of antibody and the immune status of mice. The earlier experiment of Lodmell et al. revealed that peritoneal exudate cells obtained from rabbits prevented viral plaque formation and reduced the titer of infectious virus (14). However, in their experiments, diluted antibody was added to the culture media to prevent the development of secondary plaques. It is well known that macrophages can mediate antibody-dependent cell-mediated cytotoxicity (13, 21, 23). Thus, the inhibitory activity of leukocytes observed may well have been mediated through this mechanism. To check whether antibody can enhance the inhibitory effect of macrophages, infected monolayers were incubated with the macrophages together with a 1:16 dilution of anti-HSV-2 antiserum. As can be seen in Table 2, the addition of antibody did slightly enhance the suppressive effect of macrophages on the development of viral plaques. In another study, peritoneal macrophages were obtained from animals which had been infected intraperitoneally with HSV-2 for 3 weeks. The serum neutralization titer of these mice against HSV-2 ranged from 1:160 to 1:320 when measured by 50% re-

duction of viral plaques on Vero cells. These macrophages did not exert any enhanced suppressive effect on the development of viral plaques (data not shown). Thus, the mechanisms of the inhibition of the development of viral plaques by peptone-induced macrophages are not greatly influenced by the presence of antibody, nor does the immune status of the donor animal significantly alter this macrophage function.

Age dependency of the effect. Macrophages were obtained from peptone-injected mice of various ages, and their activities were compared (Table 3). When 8-week-old DDD mice were used as the source of macrophages, a marked inhibition of viral plaques, as well as a reduction of viral yield, was obtained, as described previously. However, macrophages from 2- and 3-week-old DDD mice showed weaker functions, requiring a larger number of cells for an equivalent reduction of plaques and virus yield. In another experiment, the mortality rate of DDD mice infected with HSV-2 was found to be dependent upon the recipient's age. When animals received 5×10^3 PFU of HSV-2 (UW 268 strain) intraperitoneally, complete resistance appeared at the age of 8 weeks while mortality of 6-week-old mice fell to the neighborhood of 40%. Previously, Hirsch et al. described a similar age-dependent resistance of mice to the age-dependent maturation process of macrophages. Our results also indicated that the age dependency of the plaque-inhibiting effect of macrophages in vitro coincided with the age-dependent resistance of mice to intraperitoneal challenge with HSV-2.

To see whether this age-dependent difference simply reflects the different capacities of macrophages to support viral replication (9), macrophages obtained from mice of different ages were infected with HSV-2 at an MOI of 5. After 2 h of virus adsorption, media were removed, and the macrophage monolayer (5×10^6 cells

TABLE 2. *Effect of antibody on the inhibitory effect of macrophages*

Ratio of MEF to macrophages	No. of plaques ^a	
	With antibody ^b	Without antibody
1:0	24 ± 4.2	22 ± 4.6
1:1	22 ± 5.4	22 ± 2.8
1:5	11 ± 6.2 (<0.3)	18 ± 3.8
1:10	8 ± 3.8 (<0.1)	13 ± 5.6 (<0.3)
1:20	1 ± 1.8 (<0.001)	4 ± 2.6 (<0.05)

^a Mean number of viral plaques ± the standard error of the mean for six wells. Comparisons were made between groups by Student's *t* test, and *P* values are shown in parentheses.

^b A 1:16 dilution of rabbit antibody (neutralizing antibody titer = 2,000) was added to each well.

TABLE 3. *Age dependency of the inhibition of viral plaques by macrophages*

Ratio of MEF to macrophages	Age of mice from which activated macrophages were obtained:					
	2 weeks old		3 weeks old		8 weeks old	
	No. of plaques ^a	Virus titer ^b	No. of plaques	Virus titer	No. of plaques	Virus titer
1:0	35 ± 4.8	1×10^4	35 ± 3.9	3×10^4	36 ± 6.2	3×10^4
1:1	34 ± 5.2	1×10^4	24 ± 4.6 (<0.01)	3×10^3	27 ± 3.7 (<0.05)	6×10^3
1:5	—	—	25 ± 4.9 (<0.01)	—	—	—
1:10	18 ± 3.6 (<0.01)	2.8×10^3	24 ± 4.9 (<0.01)	5×10^3	12 ± 3.5 (<0.001)	3×10^3
1:25	20 ± 4.4 (<0.01)	—	6 ± 3.0 (<0.001)	4×10^2	4 ± 2.8 (<0.001)	1.6×10^2

^a Mean number of viral plaques ± the standard error of the mean for nine wells. Comparisons were made between groups by Student's *t* test, and *P* values are shown in parentheses.

^b Plaque-forming units per milliliter.

per well) was washed with PBS and then refed with GM. At given times thereafter macrophages were removed from the glass bottom with a rubber policeman, and the cell suspension was sonicated for 20 s with a sonicator (Chiyoda Riken Co.; 20 kc, 150 W). After the removal of all debris by centrifugation, virus titer was determined on Vero cells. As can be seen in Table 4, HSV could grow slightly in macrophages from 2- and 3-week-old mice but could not grow in those from 8-week-old mice. The results were substantiated by an immunofluorescence study. Specific fluorescence could not be seen in the macrophages except during the initial adsorption of virus onto the cell surface and in a few occasional preparations of the 2-week-old mouse macrophages.

The next experiment was designed to compare the numbers of macrophages per gram of body weight among mice of different ages. Four days after the intraperitoneal injection of 1 ml of 2% peptone, the peritoneal cavity was washed thoroughly, and the number of macrophages obtained was calculated. Eight-week-old mice yielded 10^5 cells per g of body weight, whereas the numbers of macrophages from 3- and 2-week-old mice were about 50% (5×10^4 /g of body weight) and 20% (2×10^4 /g of body weight) of that of the 8-week-old mice, respectively. Thus, there appeared to be a relationship between the age of animals and the number of macrophages capable of being mobilized to the site of stimulation. This relationship might be another factor which influences the mortality rate of animals of different ages.

Reappearance of viral plaques after removal of macrophages. It has been shown that the inhibitory effect of macrophage-rich peritoneal exudate cells was temporary and that viral plaques reappeared after removal of the exudate cells (14). To see if this was also the case in the present system, macrophages were removed after 48 h of incubation; then the monolayers were refed with GM and examined for viral plaques at 96 h. A good inhibition of plaques was obtained at 48 h when macrophages

were added at a ratio of 10:1, whereas plaques reappeared after the removal of macrophages at 96 h (Table 5).

Effect of anesthetics on the activity of macrophages. Recent evidence suggests that local anesthetics such as lidocaine and procaine can affect the phagocytic activity of macrophages (5). It is known that these cationic anesthetics can elicit an extensive rounding of macrophages and inhibit a variety of cell surface phenomena, including cell-to-substrate adhesion, locomotion, spreading, cell fusion, capping of surface immunoglobulin, and cell-mediated cytotoxicity (11, 18, 19, 22). To see whether inhibition of these cell surface phenomena by the drugs closely paralleled their effects on phagocytosis, we incubated macrophages with various concentrations of anesthetics and followed the changes in the phagocytic activity as detected by immune opsonization and particle uptake (Table 6). With 0.05% procaine, the macrophages showed an extensive rounding, and some cells detached from the glass surface. With

TABLE 5. *Effects of the removal of macrophages*

Ratio of MEF to macrophages	No. of plaques ^a			
	3 weeks ^b		8 weeks	
	48 h ^c	96 h	48 h	96 h
1:0	14	ND	12	19
1:1	11	ND	13	20
1:10	7	ND	6	12

^a Average number of plaques for six wells. ND, Not done.

^b Age of mice from which macrophages were obtained.

^c Time after infection; 48 h after infection, macrophages were removed.

TABLE 6. *Effect of procaine on the phagocytosis by macrophages^a*

Procaine concn (%)	Phagocytosis (%) of:			
	Sensitized SRBC	SRBC	Carbon	Latex particles
0.2	1	0	0	4
0.1	3	0	—	5
0.05	10	2	—	9
0.0	84	31	—	63

^a One milliliter of a 2% suspension of either hemolysin-coated or uncoated sheep erythrocytes (SRBC), colloidal carbon (0.01 mg/ml), and 0.1 ml of polystyrene latex particle ($\rho = 0.109$, 0.2% solution) was mixed with GM containing procaine in amounts calculated to produce final concentrations of 0.05 to 0.2%. Macrophages were exposed to these mixtures for 1 h at 37°C. Then they were washed, stained with Giemsa, and checked for phagocytic activities under a light and a phase-contrast microscope.

TABLE 4. *Virus growth in macrophages.*

Time after infection	Virus titer (PFU/ 10^7 cells) ^a		
	2 weeks ^b	3 weeks	8 weeks
4	0	10	10
6	5	5	10
12	0	0	0
18	10	10	0
24	20	20	0

^a Mean number of viral plaques for nine wells.

^b Age of animals from which macrophages were obtained.

increasing concentrations of the drug, many cells detached from the bottom, and their phagocytic activity was completely abolished. The next experiment was designed to see whether macrophages treated with procaine were able to inhibit the development of viral plaques. The numbers of HSV-2 plaques after 24 h of incubation are compared in Table 7. When the concentration of procaine or lidocaine exceeded 0.2%, the toxic effect of the drugs on both macrophages and MEF became evident since most of the uninfected and infected MEF which had been overlaid with macrophages were rounded and detached from the tray. Lidocaine appeared to be more toxic to MEF, and further experiments were done with 0.05 to 0.1% procaine. A macrophage suspension was mixed with procaine at doses to make the final concentration 0.05 to 0.1% and overlaid onto HSV-2-infected monolayers. As can be seen in Table 7, the macrophages treated with procaine could inhibit the development of viral plaques. When monolayers were overlaid with macrophages treated with 0.05 to 0.1% procaine, viral plaques were completely suppressed. In contrast, in monolayers overlaid with the same concentrations of procaine only, 13 to 42 viral plaques developed 24 h after infection although the number of viral plaques was decreased at the higher concentration of procaine in the overlay media. These results indicate that macrophages still retain their inhibitory activity even after being deprived of their phagocytic activity.

Assay of ⁵¹Cr release from target cells. To determine whether the inhibition of the viral plaques by macrophages stems from a cytotoxic or cytolytic event or from a nonlytic cytostasis, we labeled target cells with ⁵¹Cr and overlaid them with various numbers of macrophages. There was no significant increase in release of radioactivity from either infected or uninfected MEF overlaid with peritoneal macrophages as

TABLE 7. Effect of the procaine-treated macrophages on the development of viral plaques^a

Procaine concn (%)	No. of plaques	
	With macrophages	Without macrophages
0.2	0, 0	Cells rounded
0.1	0, 0	18, 13
0.05	0, 0	38, 42
(-)	0, 0	49, 54

^a Macrophages were suspended in procaine-containing GM in a number calculated to make the final macrophage-to-target cell ratio 20:1 and were added to HSV-infected monolayers. Controls were incubated with macrophages in the absence of the drugs, with the drugs in the absence of macrophages, and without drugs or macrophages.

compared to spontaneous release throughout the experiments (data not shown).

Effect on [¹²⁵I]IUDR incorporation. Pepitone-induced macrophages showed a remarkable inhibition of [¹²⁵I]IUDR uptake when plated onto MEF. MEF infected with UV-inactivated HSV-2 or active HSV-2 also resulted in a marked macrophage-dependent cytostasis without a preference for the virus-infected cells. In both cases, inhibition was proportional to the macrophage concentration in wells (Table 8) and was little influenced by the initial number of target cells in the culture (data not shown). It was considered that, if the macrophage function was of a cytostatic nature, serum deprivation of cells prior to infection would amplify the macrophage effect, because medium change following the infection with serum-containing fluid could stimulate new deoxyribonucleic acid synthesis. When confluent cultures of MEF were maintained for 24 h in serum-deprived medium before the test of [¹²⁵I]IUDR incorporation, a greater inhibition of [¹²⁵I]IUDR uptake was observed (Table 9), supporting the assumption that macrophage-mediated cytostasis is the basis of inhibition of viral plaques.

DISCUSSION

The importance of macrophages in the host defense system has been implied by many pre-

TABLE 8. Inhibition by macrophages of [¹²⁵I]IUDR incorporation in normal and HSV-2-infected MEF

Target cell	^[125] IUDR incorporation in targets (cpm) ^a	% ^[125] IUDR incorporation ^b with macrophage-to-target ratio of:		
		150:1	100:1	50:1
Expt 1				
MOI = 1.0				
MEF	78,723 ± 3,030	5.3	9.5	28.8
MEF-HSV	33,952 ± 6,432	28.3	70.0	102.0
MEF-UV-HSV	57,941 ± 1,102	2.9	12.8	41.0
Expt 2				
MOI = 0.1				
MEF-HSV	74,838 ± 7,957	4.9	19.5	52.8
MEF-UV-HSV	53,304 ± 7,194	4.2	10.3	45.8

^a In each well, 10⁴ target cells were seeded, and HSV-2 or UV-inactivated HSV-2 (UV-HSV) was adsorbed for 2 h. After repeated washings, the monolayers were incubated with macrophages for 18 h and [¹²⁵I]IUDR was added. After a further 6 h of incubation, the samples were counted. Results show the mean ± the standard error of the mean.

^b [¹²⁵I]IUDR incorporation into macrophages void of targets = 331 ± 56 cpm. Percentage incorporation was calculated after subtraction of [¹²⁵I]IUDR incorporation in macrophages void of targets. Standard error of the mean < 10%.

TABLE 9. *Effect of serum deprivation on inhibition by macrophages of [¹²⁵I]IUDR incorporation in virus-infected MEF*

Target	[¹²⁵ I]IUDR incorporation in targets (cpm) ^a	% [¹²⁵ I]IUDR incorporation ^b with macrophage to target ratio of:		
		150:1	100:1	50:1
MEF cultured with serum-deprived medium ^c				
MEF	94,590 ± 2,837	5.6	20.3	27.0
HSV-2	38,080 ± 1,515	5.7	55.3	83.5
UV-HSV-2	113,630 ± 5,670	5.3	11.8	33.8
MEF cultured with serum-supplemented medium				
MEF	63,911 ± 4,130	8.1	29.6	47.2
HSV-2	34,017 ± 510	44.6	71.8	97.2
UV-HSV-2	63,085 ± 2,286	10.9	19.2	98.0

^a Target cells (10⁴ per well) were seeded and incubated for 24 h. They were then infected with HSV-2 or UV-inactivated HSV-2 (UV-HSV-2) for 2 h and washed, and the monolayers were incubated with macrophages for 18 h. After 6 h of incorporation of [¹²⁵I]IUDR, the samples were counted. Results show the mean ± the standard error of the mean.

^b [¹²⁵I]IUDR incorporation into macrophages void of targets = 434 ± 64 cpm. Percentage of incorporation was calculated after subtraction of [¹²⁵I]IUDR incorporation in macrophages void of targets. Standard error of the mean < 10%.

^c MEF were cultured for 24 h in serum-deprived minimal essential medium before the virus infection.

vious reports, based on the unusual susceptibility of animals to viral infections accompanying maturational or acquired defects of macrophage-mediated immunity and the role played by macrophages in restricting viral spread by antibody-dependent cellular cytotoxicity or immune opsonization (3, 9, 13, 20, 21, 23-29).

In HSV infection, it is known that virus can spread to neighboring cells directly through intercellular bridging or cell fusion without being exposed to the extracellular fluid. Previously, Lodmell et al. showed that macrophage-rich peritoneal leukocytes of nonimmune rabbits when coupled with anti-HSV antibody curbed the spread of HSV infection *in vitro* (14). In the present study, we determined the cell responsible for this effect to be the macrophage by a more precise analysis. In contrast to the previous experiments in which antibody against HSV was present in the media, we now could stop HSV spread by peptone-induced peritoneal macrophages in the absence of antibody. This suggests that, in the present system, macrophages might be able to exert their defensive function through mechanisms other than mediation of antibody-dependent cellular cytotoxicity or phagocytosis.

In this study, tryptose peptone was used to induce macrophages. Whether the macrophage

induction by this nonspecific stimulation is functionally the same as the induction occurring after actual infection is not known. Rodda and White reported that viral infections were far more provocative in this respect than intraperitoneal inoculation of casein hydrolysate (20). Therefore, it might be reasonable to suppose that macrophages mobilized in an actual infection site might be more effective in halting HSV spread.

The age-dependent susceptibility to HSV infection has been well documented by earlier investigators (8, 9, 12, 26), and our present results reconfirmed it. It appears that macrophage maturation is a factor contributing to this age-dependent resistance since transfer to syngeneic adult mice of silica or anti-macrophage serum allowed lethal infection to occur (28). Johnson reported that macrophages from newborn mice could support the growth and spread of HSV *in vitro*, whereas those from adult mice could not. This suggests that these differences of macrophages could account for lethal infection in newborn mice and resistance of adult mice to HSV infection (9). In the present study, too, growth of HSV in macrophages from adults and from newborns *in vitro* showed differences: macrophages from newborns supported some HSV replication, whereas those from adult mice did not appreciably support replication. However, it appears from the present study that not only a maturational difference exists but also the number of macrophages which can be mobilized to the infection site is age dependent, suggesting that a certain number of macrophages are needed to protect the host from virus infection and that this quantitative difference also serves as a factor in the age-dependent resistance.

The use of a low concentration of procaine clearly cuts off the phagocytic activity of macrophages as seen by the inhibition of ingestion of particulate matter and rosette formation of sensitized sheep erythrocytes (5). These procaine-treated macrophages still possessed the inhibitory effect on HSV spread although procaine itself has some inhibitory effect on the development of viral plaques, perhaps by causing a gap between adjacent monolayer cells at high concentrations. These results might suggest that macrophages exert their plaque-inhibiting effect on infected cells through functions different from their phagocytic activity.

Recent studies have demonstrated that peritoneal exudate cells can inhibit target cell growth. Goldman and Hogg reported that this inhibitory effect of peritoneal exudate cells is mediated primarily by the cytostatic effect and not by the cytolytic effect of macrophages on

target fibroblast (6). Moreover, they showed marked enhancement of cytostasis when target cells were infected with murine sarcoma virus, murine leukemia virus, Sendai virus, or influenza virus. In the present study, we could not demonstrate a significant ^{51}Cr release from target cells, and this suggests that the strong inhibitory effect of peptone-induced macrophages on [^{125}I]IUDR incorporation into target cells is not coupled to target cell lysis. Although we could not demonstrate an enhanced susceptibility of HSV-infected target cells to cytostasis, this might be due to differences among the individual virus-host systems.

In the present study, HSV plaques reappeared after the removal of macrophages. These results also indicate that the macrophage action is not cytotoxic but rather is cytostatic and does not destroy monolayer cells since these cells can support HSV replication again after regaining favorable conditions. This fact argues against the objection that the macrophage function stated in the present experiments simply reflects a nutritional disturbance of the underlying monolayer cells.

In this connection, another point of importance relevant to the host defense mechanism is that macrophages alone are not sufficient to cure the viral infection, but the actual combat with the virus also requires a complete set of other host defense mechanisms, such as effector T cells, antibody-dependent cellular cytotoxicity mediated by K cells, humoral antibodies, and such mediators as interferon (13, 15, 29).

In vivo, the host defense mechanisms against viral infection are a mixture of specific immunological reactions and the nonspecific factors mobilized during the infection (16). This paper does not include any experiment on the interaction between macrophages and effector T cells nor any evidence for the relevance of the in vivo function of activated macrophages in eliminating HSV infection. Further study on the lymphocyte-macrophage interaction which might give rise to a more effective way of activating macrophages for viral infection and a proper evaluation of their in vivo role will have to follow in the future.

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