

# Herpes Simplex Virus-induced Suppression of Macrophage-mediated Tumoricidal Activity in Murine Macrophages<sup>1</sup>

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## ABSTRACT

Herpes simplex virus (HSV) infections can enhance the progression of neoplastic diseases. Since macrophages can be activated to become tumoricidal and may figure prominently in host defense against cancer, the ability of HSV to modify macrophage-mediated tumoricidal functions was evaluated. Murine peritoneal macrophages treated with HSV could not be activated to a tumoricidal state by mouse recombinant  $\gamma$ -interferon (IFN- $\gamma$ ). Addition of HSV 4 h after treatment with IFN- $\gamma$ , at a time when the macrophages are fully committed to developing the cytotoxic phenotype, blocked macrophage-mediated lysis of syngeneic melanoma target cells. This inhibition of activation and cytotoxicity was not due simply to uptake of virus particles, because treatment with heat-inactivated HSV at 4-h posttreatment with IFN- $\gamma$  had no effect. In addition, HSV did not undergo a productive infection within macrophages, suggesting that the observed inhibitory activity might be due to a virus-induced product. In this regard, treatment of macrophages with recombinant  $\alpha$ -interferon suppressed the activation of these cells by IFN- $\gamma$ , suggesting that virus-induced  $\alpha$ -interferon may be mediating all or part of the suppressive activity. These studies suggest that enhancement of tumor progression following HSV infection may be related to the virus-induced suppression of macrophage-mediated tumoricidal activity.

## INTRODUCTION

HSV<sup>3</sup> infections increase in frequency and severity in episodes of malignancy, organ transplantation, or immunosuppression (1-3). In addition, these viruses can enhance primary tumor growth and metastasis (4, 5). The mechanisms through which HSV may affect the pathogenesis of neoplastic diseases are unclear, although suppression of T-lymphocyte responses (6) and promotion of tumor growth via HSV-induced growth-stimulating factors has been suggested (7).

Recent evidence demonstrates that cells of the monocyte-macrophage series are important elements in the host defense against cancer (8, 9). Macrophages may be activated by a number of immunomodulatory substances to a cytotoxic state, enabling them to selectively bind and lyse tumor cells without harming normal cells (10, 11). The recognition of macrophage-mediated cytotoxicity of tumor cells prompted Hibbs *et al.* (12) to propose a possible surveillance mechanism for macrophages against nascent cancer cells. Since HSV binds to and is rapidly internalized by macrophages (13), and under certain conditions may replicate within these cells (14), we sought to determine whether HSV affects the capacity of macrophages to become activated to a tumoricidal state. We report here that HSV suppressed the ability of IFN- $\gamma$  to activate macrophages to the tumoricidal state; addition of HSV to macrophages 4 h after

activation with IFN- $\gamma$  completely blocked macrophage-mediated lysis of tumor cells; HSV-induced suppression of macrophage function occurred in the absence of significant virus replication; and IFN- $\alpha$ , which is produced following incubation of HSV with macrophages (15), caused a similar suppression of macrophage tumoricidal activity, suggesting a possible mechanism of action for the HSV-induced suppression. In total, these findings suggest that enhancement of tumor development by HSV may be due in part to the virus-induced suppression of macrophage-mediated lysis of tumor cells.

## MATERIALS AND METHODS

**Animals.** Specific pathogen-free 6- to 8-week-old male mice of the inbred strain C57BL/6  $\times$  C3H F<sub>1</sub> (B6C3F<sub>1</sub>) were obtained from the NCI-Frederick Cancer Research Facility animal production area (Frederick, MD).

**Medium.** Eagle's minimum essential medium was supplemented with 5% FBS, sodium pyruvate, nonessential amino acids, gentamicin, L-glutamine, and 2 $\times$  vitamins (CMEM) and was obtained from M.A. Bioproducts (Walkersville, MD). Medium and FBS used in these studies were endotoxin negative as determined by the *Limulus* ameobocyte lysate assay (16).

**Virus.** HSV-1 strain 14012 was plaque purified, propagated in Vero cells (17), and stored at -80°C. Viruses were titrated in Vero cells using a methylcellulose overlay. Briefly, confluent monolayers of Vero cells were treated with serial 10-fold dilutions of virus samples. Following a 2-h adsorption, inoculum was removed and replaced with 0.5% methylcellulose supplemented with 5% FBS and antibiotics. Plates were incubated for 72 h, after which the cells were fixed in methanol and stained with Giemsa. Plates were dried and plaques were counted with a plaque counter. Data were expressed as plaque-forming units per ml.

**Isolation of Peritoneal Macrophages.** Thioglycollate-elicited peritoneal macrophages were obtained from mice as previously described (18). Briefly, mice were given injections i.p. of 1.5 ml thioglycollate broth (BBL Microbiology Systems, Cockeysville, MD), and peritoneal exudate cells were harvested by lavage 4 to 5 days later with Ca<sup>2+</sup>-Mg<sup>2+</sup>-free HBSS. Cells were plated in 96-well flat-bottom microtiter dishes (Falcon Plastics, Oxnard, CA) at 1  $\times$  10<sup>5</sup> cells/well in serum-free CMEM and allowed to adhere for 1 h at 37°C in a 5% CO<sub>2</sub> and air atmosphere. Nonadherent cells were washed out and greater than 95% of the adherent cells were determined to be macrophages by morphological, biochemical, and functional examination (19). When macrophages were treated with HSV-1, no differences were observed between control and infected cells with regard to either macrophage adherence or viability.

**Activation of Peritoneal Macrophages.** Macrophages were treated with CMEM or mouse recombinant IFN- $\gamma$  (generously supplied by Genentech, Inc., South San Francisco, CA) and lipopolysaccharide (*Salmonella typhimurium*; Difco Laboratories, Detroit, MI) at 10 ng/ml was included as a second signal for activation (20). HSV or recombinant IFN- $\alpha$  A/D (generously supplied by Hoffmann La Roche Inc., Nutley, NJ) was added to cultures at times indicated in the table legends. Following a 24-h incubation period with IFN- $\gamma$ , plates were prepared for cytotoxicity assays.

**Target Cells for Cytotoxicity Assays.** The B16-F10 cell line derived from a spontaneous melanoma of C57BL/6 origin (21) was used as a target for macrophage cytotoxicity assays. Cells were cultured in CMEM supplemented with 10% FBS.

**Macrophage-mediated Cytotoxicity Assay.** Lysis of B16-F10 tumor

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<sup>3</sup> The abbreviations used are: HSV, herpes simplex virus; IFN, interferon; IFN- $\gamma$ ,  $\gamma$ -interferon; IFN- $\alpha$ ,  $\alpha$ -interferon; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; CMEM, complete minimum essential medium.

cells by activated macrophages was evaluated by using a previously described cytotoxicity assay (22). Briefly, target cells in exponential growth phase were incubated 24 h in CMEM plus 10% FBS supplemented with [<sup>125</sup>I]iododeoxyuridine (0.3 μCi/ml; specific activity, 200 mCi/μmol; New England Nuclear, Boston, MA). The cells were washed once with HBSS to remove unincorporated label, harvested by trypsinization (0.25% trypsin and 0.02% EDTA for 1 min at room temperature), resuspended in CMEM, and plated at 1 × 10<sup>4</sup> cells/well in wells containing macrophage monolayers. Tumor cells in CMEM were also added to empty wells for control determinations of plating efficiency. Spontaneous cytotoxicity mediated by macrophages was monitored by cultures of tumor cells and macrophages in CMEM. Twenty-four h after the addition of labeled target cells, the cultures were refed with CMEM and incubated an additional 48 h at 37°C in a 5% CO<sub>2</sub> and air atmosphere. At assay termination, cultures were washed twice in HBSS and lysed with 0.1 ml of 0.1 N NaOH. The lysate was absorbed onto cotton swabs and placed in 12- x 75-mm tubes and counted in a gamma counter. The macrophage-mediated cytotoxicity percentage was calculated as follows:

$$\% \text{ of cytotoxicity} = 100 \times (A - B) + A$$

where *A* is cpm in tumor cells with control macrophages, and *B* is cpm in tumor cells cultured with experimental macrophages.

**Virus Production Assays.** Peritoneal exudate cells (1 × 10<sup>6</sup>/ml) were plated into 24-well Costar (Cambridge, MA) culture dishes at 0.5 ml/well and incubated for 1 h at 37°C. Nonadherent cells were washed out, and monolayers of macrophages were inoculated with HSV-1 at a multiplicity of infection equal to 1.0 and incubated for 2 h at 37°C. In some experiments, HSV-1 was not added until 24 h posttreatment with IFN-γ in order to evaluate the capacity of activated macrophages to support a productive viral infection. Following virus adsorption, cultures were incubated in CMEM at 37°C in a 5% CO<sub>2</sub> in air atmosphere for 48 h, supernatants were removed, frozen at -80°C, and assayed for virus by plaque assay.

**Statistics.** Student's *t* test (two-tailed) was used to evaluate the significance of observed differences between experimental and control groups.

**RESULTS**

Treatment of murine peritoneal macrophages with HSV-1 suppressed the capacity of IFN-γ to activate these cells to a tumoricidal state (Table 1). In these experiments, virus was added to macrophage monolayers 1 h before the addition of IFN-γ. Since large quantities of virus can theoretically coat the macrophage surface and thus inhibit IFN-γ receptor interactions, another series of studies was conducted to evaluate the effect of HSV on macrophages previously activated and com-

mitted to the cytotoxic phenotype. Table 2 demonstrates that HSV-1 added 4 h after treatment of IFN-γ on murine macrophages completely shut down the tumoricidal capacity of these cells. Control studies (Table 2) indicated that 4-h treatment with IFN-γ was sufficient to commit the macrophages to a cytotoxic phenotype, which concurs with previously described studies (23).

Heat treatment of HSV-1 for 1 h at 56°C inactivated the virus and blocked its capacity to modulate macrophage function (Table 3). In addition, treatment of macrophages with active HSV-1 produced low levels of IFN (100 units/ml), while heat-inactivated virus produced insignificant levels of IFN. Macrophage-mediated IFN was characterized as IFN-α by appropriate control neutralization assays with anti IFN-α antibodies (data not shown). Thus, it appears that an active virus infection is required for the HSV-1 modification of macrophage-mediated tumor cytotoxicity.

HSV-1 did not cause a productive infection in either quiescent or activated macrophages (Table 4). As a result, we reasoned that a virus-induced product might be modifying the ability of macrophages to be activated by IFN-γ to the tumoricidal state. Since HSV stimulated the production of IFN-α in our system and in similar systems by others (15, 24), we sought first to evaluate the potential suppressive activity of IFN-α. Recombinant IFN-α A/D, a human hybrid interferon that demonstrates antiviral activity in both human and murine systems (25), completely blocked the capacity of IFN-γ to activate murine macrophages to a tumoricidal state (Table 5), suggesting

Table 2 Inhibition of macrophage-mediated tumoricidal activity by HSV

Macrophages were treated with CMEM or IFN-γ (10 units/ml) for 24 h prior to addition of target cells as described in "Materials and Methods." Effector:target cell ratios were 10:1.

Macrophage treatment	HSV treatment <sup>a</sup>	% of cytotoxicity <sup>b</sup>
CMEM		-1.3
CMEM	<i>t</i> = 0	-2.5
CMEM	<i>t</i> = 4	-0.8
IFN-γ		31.6 <sup>c</sup>
IFN-γ	<i>t</i> = 0	0.8
IFN-γ	<i>t</i> = 4	-5.0
IFN-γ 4 h, CMEM 20 h		29.1 <sup>d</sup>

<sup>a</sup> Macrophages were infected with HSV-1, multiplicity of infection of 1.0, at *t* = 0 or *t* = 4 h after treatment with IFN-γ. Unadsorbed virus was rinsed from the wells after a 1-h incubation, and cultures were refed with CMEM or IFN-γ for the duration of the activation period.

<sup>b</sup> Percentage of cytotoxicity indicates lysis of [<sup>125</sup>I]iododeoxyuridine-labeled target cells as described in "Materials and Methods." Values are from a representative of three replicate experiments.

<sup>c</sup> *P* < 0.01 versus CMEM.

<sup>d</sup> Macrophages were treated with IFN-γ for 4 h, the IFN-γ was washed out and replaced with CMEM for the remaining 20 h prior to addition of target cells.

Table 1 Suppression of IFN-γ-induced macrophage activation by HSV-1

Macrophage activation was monitored by the macrophage-mediated tumor cytotoxicity assay as described in "Materials and Methods." Effector:target ratios were 10:1.

Macrophage treatment <sup>a</sup>	HSV treatment <sup>b</sup>	<sup>125</sup> I dUrd (cpm) <sup>c, d</sup>
No macrophages, tumor cells alone	-	1337 ± 74 <sup>e</sup>
CMEM	-	1339 ± 31
CMEM	+	1360 ± 15
IFN-γ	-	753 ± 103 (43.8) <sup>f</sup>
IFN-γ	+	1286 ± 30 (3.9) <sup>g</sup>

<sup>a</sup> Macrophages were treated with CMEM or IFN-γ (100 units/ml) for 24 h prior to addition of target cells as described in "Materials and Methods."

<sup>b</sup> One h prior to the addition of IFN-γ, macrophages were infected with HSV-1 at a multiplicity of infection of 1.0. Unadsorbed virus was removed by washing 3 times with CMEM, and CMEM or IFN-γ was added as described above.

<sup>c</sup> IdUrd, iododeoxyuridine.

<sup>d</sup> Counts indicated residual <sup>125</sup>I dUrd retained in viable tumor cells following harvest of cytotoxicity assay at 72 h after cocultivation with macrophages.

<sup>e</sup> Mean cpm ± SD. All samples were assayed in triplicate.

<sup>f</sup> Numbers in parentheses, percentage of cytotoxicity. These data are representative of four replicate experiments.

<sup>g</sup> *P* < 0.01 versus IFN-γ-treated macrophages.

Table 3 Effect of heat-inactivated HSV on IFN-γ-induced macrophage-mediated cytotoxicity

Macrophages were treated with CMEM or IFN-γ (100 units/ml) as described in Table 1. At *t* = 4 hr, 20 μl of HSV-1 or HSV-1 heat-inactivated at 56°C for 1 h were added to culture wells for the duration of the activation period. Effector:target ratios were 10:1.

Macrophage treatment	% of cytotoxicity <sup>a</sup>
CMEM	-1.8
IFN-γ	50.9
CMEM + HSV	0.8
IFN-γ + HSV	1.7 <sup>b</sup>
CMEM + heat-inactivated HSV	-0.6
IFN-γ + heat-inactivated HSV	39.8 <sup>c</sup>

<sup>a</sup> Percentage of cytotoxicity indicates lysis of [<sup>125</sup>I]iododeoxyuridine-labeled target cells as described in "Materials and Methods." Values are from a representative of three replicate experiments.

<sup>b</sup> *P* < 0.001 versus IFN-γ.

<sup>c</sup> Not significant versus IFN-γ.

**Table 4** Lack of replication of HSV in normal and activated macrophages  
Macrophages were treated with CMEM, IFN- $\gamma$  (100 units/ml), or lipopoly-saccharide (1  $\mu$ g/ml) for 24 h.

Macrophage treatment	HSV treatment <sup>d</sup>	PFU/ml <sup>b, c</sup>
CMEM	$t = 0$	$4.0 \times 10^2$
CMEM	$t = 24$	$1.9 \times 10^2$
IFN- $\gamma$	$t = 0$	$7.6 \times 10^2$
IFN- $\gamma$	$t = 24$	$7.3 \times 10^2$
LPS	$t = 0$	$3.5 \times 10^2$
LPS	$t = 24$	$3.2 \times 10^2$
Vero cell control <sup>d</sup>		$1.2 \times 10^6$

<sup>a</sup> HSV-1 was added to macrophage cultures at the same time ( $t = 0$ ) or 24 h ( $t = 24$ ) after activation at a multiplicity of infection of 1.0. Cultures were incubated for 48 h prior to harvest of supernatant for virus titration.

<sup>b</sup> PFU, plaque-forming units; LPS, lipopolysaccharide.

<sup>c</sup> PFU/ml indicates plaque-forming units/ml by a methylcellulose plaque assay of Vero cells.

<sup>d</sup> Growth of HSV-1 in Vero cells alone is given for comparative purposes.

**Table 5** Inhibition of macrophage-mediated tumoricidal activity by IFN- $\alpha$   
 $\alpha$ -interferon used was recombinant IFN- $\alpha$  A/D (Hoffmann La Roche Inc.).

Macrophage treatment <sup>a</sup>	<sup>125</sup> I dUrd (cpm) <sup>b</sup>
No macrophages, tumor cells alone	$2967 \pm 193^c$
CMEM	$2870 \pm 120$
IFN- $\gamma$	$966 \pm 125 (66.4)^d$
IFN- $\alpha$	$3062 \pm 23 (-6.7)$
IFN- $\gamma$ + IFN- $\alpha$	$2917 \pm 312 (-1.6)^e$

<sup>a</sup> Macrophages were treated with CMEM, IFN- $\gamma$  (100 units/ml); IFN- $\alpha$  (100 units/ml), or IFN- $\gamma$  plus IFN- $\alpha$  (both at 100 units/ml). IFN- $\alpha$  was added 2 h after treatment with IFN- $\gamma$ . Activators remained on the macrophages for 24 h prior to addition of [<sup>125</sup>I]iododeoxyuridine labeled tumor cells for cytotoxicity assay. Effector:target cell ratios were 10:1.

<sup>b</sup> IdUrd, iododeoxyuridine.

<sup>c</sup> Mean cpm  $\pm$  SD as described in Table 1.

<sup>d</sup> Numbers in parentheses, percentage of cytotoxicity. These data are representative of five replicate experiments.

<sup>e</sup>  $P < 0.001$  versus IFN- $\gamma$ .

that HSV-1-induced IFN- $\alpha$  may be involved in the suppression of macrophage activation to a cytotoxic state. Kinetic analyses of the regulatory effects of recombinant IFN- $\alpha$  A/D are described elsewhere (26).

## DISCUSSION

These studies have shown that HSV can modify the cytotoxic properties of macrophages by both blocking the capacity for immunomodulator-mediated activation and by turning off macrophages previously committed to the tumoricidal phenotype. The observation that infectious virus was required for HSV-induced suppression coupled with the lack of productive infection by HSV in these macrophages suggested that a virus-induced product might be responsible for the inhibitory activity. Treatment of macrophages with IFN- $\alpha$  caused a similar suppression of macrophage tumoricidal activity, suggesting that HSV-induced IFN- $\alpha$  may be involved in modifying macrophage cytotoxic functions. These studies thus suggest that HSV-induced suppression of macrophage-mediated tumoricidal activity may be a major factor in the enhancement of primary tumor growth and metastasis observed during concomitant herpes infections.

Macrophages play a central role in the host defense against HSV infections (27). Severe systemic neonatal infections with HSV have been correlated with the ability of HSV to replicate in macrophages from newborn but not from adult mice (28). Similarly, adult macrophages adoptively transferred to newborn mice confer resistance against HSV in the newborn (29). Agents, such as silica, trypan blue, carageenan, and dextran sulfate that suppress macrophage function enhance the course of herpetic infection in murine model systems (30, 31). Con-

versely, activation of macrophages by immunomodulators can lead to prophylaxis of lethal HSV in mice (32, 34). Thus, the interaction of HSV with macrophages is important to the pathogenesis of these infections. It is important to note, however, that other cellular immune effector functions, including T-cells and natural killer cells, have been implicated in the host response to HSV (35), and interactions of HSV with these cell types may also be important. Although several studies indicate that macrophages are not highly permissive to HSV unless cultured for several days *in vitro* (14, 36), these viruses do cause abortive cycles of replication and can induce interferon (15, 24). As a result, several important macrophage functional activities may be modulated by HSV infection in the absence of productive replication cycles. Plaeger-Marshall and Smith (6) demonstrated that exposure of rabbit alveolar and peritoneal macrophages to infectious HSV markedly suppressed phagocytic activity and antibody-dependent cellular cytotoxicity. Heat-inactivated virus was unable to inhibit either macrophage function. These findings concur closely with our own observations that infectious but not heat-inactivated HSV was required for modulation of macrophage-mediated tumoricidal activity. When the multiplicity of infection was varied, suppression of tumoricidal activity was observed down to a multiplicity of infection of approximately 0.4. HSV can also suppress the mitogenic responses of lymphocytes to phytohemagglutinin. It has been suggested, based on studies in both poliovirus and HSV systems, that suppression of the mitogenic response may not be caused by a direct effect on lymphocytes, but by an indirect effect on production of interleukin-1 via macrophages known to be required in the lymphoproliferative response (35).

Formation of a primary syngeneic methylcholanthrene-induced spindle cell myosarcoma was stimulated by concomitant HSV infection (4). Similarly, infection with HSV-1 and -2 accelerated primary Lewis lung tumor formation in mice and enhanced the number and size of lung metastases (5). These murine tumor model systems support the epidemiological and clinical data that indicate the bidirectional relationship between herpes and cancer, *i.e.*, that HSV increases in frequency during episodes of malignancy and that tumor enhancement occurs during concomitant HSV infections (1-5). Interestingly, Gazdar *et al.* (37) observed that IFN- $\alpha$  can enhance tumor growth using a virus-induced sarcoma system in mice. The fact that macrophages produce IFN- $\alpha$  in response to a variety of virus infections (15, 24), coupled with the lack of efficacy of heat-inactivated virus in modulating macrophage function, prompted us to examine whether IFN- $\alpha$  could suppress macrophage tumoricidal activity. Since IFN- $\alpha$  was added to macrophages after a 2-h pretreatment with IFN- $\gamma$ , the inhibitory effect of IFN- $\alpha$  could not be due to competition for cellular receptors for interferon. In this regard, recent findings indicate that IFN- $\alpha$  and IFN- $\gamma$  have independent cellular receptor sites on all cell types thus far examined (38, 39). Moreover, recent studies indicate that IFN- $\alpha$  can suppress the induction of Ia antigens on IFN- $\gamma$ -treated macrophages, thus demonstrating another inhibitory effect of IFN- $\alpha$  on IFN- $\gamma$ -macrophage interaction (40). Finally, IFN- $\alpha$  can suppress the maturation of human peripheral blood monocytes (41), suggesting that observations in the murine system may have counterparts in the human monocyte system.

The capacity of HSV interaction with macrophages to suppress macrophage tumoricidal activity, coupled with the prominent role of macrophages in both the pathogenesis of HSV infections and neoplasms, suggests that modulation of macrophage function may be implicated in the enhancement of tumor

progression by HSV. Recently, we described the use of liposome-encapsulated immunomodulators to protect mice against HSV infections by activation of macrophages *in vivo* (34). In the context of the present studies, the use of HSV-specific antivirals such as acyclovir directed to macrophages *in vivo* to suppress the modulation of macrophage function by HSV warrants investigation in tumor-bearing hosts.

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## Herpes Simplex Virus-induced Suppression of Macrophage-mediated Tumoricidal Activity in Murine Macrophages

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