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Deficient Responses of Pulmonary Macrophages from Healthy Smokers to Antiviral Lymphokines in Vitro

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The antiviral function of pulmonary macrophages obtained by broncholavage of healthy smokers and nonsmokers was studied. Compared with nonsmokers' cells, smokers' macrophages produced significantly more virus during in vitro infection with herpes simplex virus type 1 (HSV-1). Exposure of macrophages to either antiviral macrophage-activating factor or interferon-γ for 20 hr before infection resulted in diminished production of HSV-1 by both types of macrophages. However, in contrast to smokers' cells, exposure of nonsmokers' macrophages to these antiviral lymphokines totally prevented viral replication. This difference could not be attributed to diminished adsorption of virus to smokers' macrophages or to an increased proportion of extracellular to intracellular virus in smokers' cell cultures. The effect of smoking on viral infectivity did not appear to be mediated by secretion of a soluble factor by the macrophage because incubation of nonsmokers' cells with supernatant from smokers' cell cultures did not affect the growth of HSV-1.

Cigarette smoking increases both the risk and severity of viral infection of the respiratory tract. Healthy young adult smokers have more frequent acute infections of the respiratory tract than do nonsmokers of the same age [1, 2]. During episodes of acute nonspecific illness in the respiratory tract, smokers report more symptoms [3] and develop more signs [4] in the lower respiratory tract than do nonsmokers. Although the etiology of acute illness in the respiratory tract was not characterized in some of these studies, a substantial proportion of acute nonspecific illness in the respiratory tract is caused by viral infection [5].

Smoking has been found to influence the impact of respiratory tract infection on the host in studies in which definite viral etiology has been specified. During an epidemic of influenza (H1N1) in young soldiers, smoking was associated with increased risk of development of clinically apparent infection as well as increased likelihood of a severe influenza syndrome [6]. In chronic bronchitis in which smoking is the major causative factor [7], rates of viral infections in the respiratory tract are greater in smoking than in nonsmoking controls for rhinoviruses [8], respiratory syncytial virus [9], influenza A and B viruses, parainfluenza virus types 1, 2, and 3, and coronavirus OC 43 [9, 10]. Viral infection of the respiratory tract is also responsible for substantial morbidity in chronic bronchitis; viruses have been implicated in up to one-third of all exacerbations of bronchitis [11, 12].

In spite of the impact of cigarette smoking on viral infection of the respiratory tract, little is known about how smoking alters respiratory defenses against viruses. The pulmonary macrophage is a principal cellular defense in the lung against infectious agents, including viruses [13, 14]. Because various functional, metabolic, and structural alterations have been demonstrated in this cell as a result of exposure to tobacco smoke, it is reasonable to speculate that certain critical antiviral functions of the pulmonary macrophage may also be affected by smoking. For this reason we assessed the infectivity of herpes simplex virus type 1 (HSV-1) for pulmonary macrophages from healthy smokers and nonsmokers. Because macrophage mechanisms for restricting viral growth may be activated in vivo by lymphokines, soluble mediators of the immune response [15, 16], we also investigated the possibility that smoking affects lung defenses by altering macrophage responsiveness to antiviral lymphokines.

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Materials and Methods

Source of macrophages. Pulmonary macrophages were harvested from healthy smokers and nonsmokers as described previously [17]. All volunteers for this study were screened by standardized questionnaire [18], chest auscultation, and spirometry [19]. Individuals were excluded from this study if they had a history or evidence of chronic pulmonary disease, abnormal lung sounds, or abnormal spirometry. To ensure that macrophages were free of respiratory viruses and had not been recently exposed to endogenous antiviral mediators such as interferon, we only accepted volunteers for broncholavage if they were free of all respiratory tract symptoms for the prior two weeks. All smokers identified in this fashion smoked at least one pack of cigarettes per day (range, one to 2.5 packs per day) for two years (range, two to 12 years).

Because preliminary studies confirmed the work of Daniels et al. [20], which demonstrated that monocytes obtained from HSV-1–seropositive and –seronegative donors were equally permissive for viral replication, the serological status of the donor was not taken into account in these studies.

Macrophage infection. Pulmonary macrophages were isolated from broncholavage fluid by centrifugation at 100 *g* for 10 min at 20 C. They were maintained in RPMI-1640 medium supplemented with 25 m*M* HEPES buffer, 50 μg of gentamicin/ml, 2 m*M* L-glutamine, and 10% heated-inactivated fetal calf serum. Cells (5×10^5) were seeded in 35-mm plastic dishes with 2-mm grids (Miles Laboratories, Naperville, Ill) to facilitate counting. Cells remaining adherent after incubation for 3 hr were >97% viable, as assessed by ability to exclude trypan blue, and >94% phagocytic of latex beads 1.1 μm in diameter. Cells from smokers and nonsmokers were similar with respect to viability and phagocytic ability. However, smokers' cells exhibited morphological differences from nonsmokers' cells by light microscopy: smokers' cells appeared larger, spread more rapidly on plastic, and contained numerous refractile intracytoplasmic inclusions.

Within 24 hr of isolation, macrophages were infected with KOS strain HSV-1 at an moi of 20:1 by exposure of cells to virus (0.1 ml) for 60 min at 37 C in an atmosphere of 95% room air and 5% CO_2. Unadsorbed virus was removed by three washes with media.

At intervals after infection, virus present in the supernatant of macrophage cultures was quantitated by determination of the number of viral pfu in replicate cultures of Vero cells [21]. In this assay 1% pooled human serum was used as a source of HSV-1–specific antibody to neutralize virus released into the culture medium after lysis of infected cells and to prevent spread of released virus to noninfected cells.

Adsorption of HSV-1 to macrophages was determined after exposure of cells to viral inoculum for 1 hr, followed by extensive washing of cells with serum-free medium to remove unadsorbed virus. Adsorbed virus was then quantitated by freeze-thawing cells at −70 C and measurement of the number of viral pfu in the lysate. Freeze-thawing cells in this manner resulted in complete disruption of macrophage cultures (as seen by light microscopy) from both smokers and nonsmokers. Cell-associated virus was quantitated at intervals after infection in a fashion analogous to the method used for determination of initial adsorption of HSV-1 to cells.

Cells were quantitated by counting pulmonary macrophages remaining on one of the four central grids of the 35-mm dish. This technique was feasible because all nonadherent cells were nonviable. Virus-specific cytolysis was determined as described previously [22] by comparison of the number of remaining cells in infected cultures at intervals after infection to those present in infected wells at 1 hr after exposure to virus. The latter number was multiplied by the percentage of cytolysis in uninfected cells at the time under analysis to account for nonspecific cell death in macrophage cultures.

Modification of infection in nonsmokers' cells by exposure to supernatant from cultures of smokers' macrophages was attempted by incubation of nonsmokers' cells with supernatants (50% by volume) from one- to three-day-old cultures of smokers' cells for 24 hr before viral infection.

Preparation of lymphokines and macrophage activation. Human interferon-gamma (IFN-γ) was obtained from Meloy Laboratories (Springfield, Va). Antiviral macrophage-activating factor (AV-MAF) was prepared from concanavalin A (10 μg/ml)–stimulated human leukocyte concentrates obtained as a by-product of plateletpheresis as described previously [23]. This procedure involves partial purification of leukocyte supernatants by gel filtration on a column (2.5 × 100 cm) of Sephadex® G-100 (Pharmacia Fine Chemicals, Piscataway, NJ), followed by isoelectric focusing of the effluent on ampholine

electrofocusing equipment (model 8102; LKB Producter, Bromma, Sweden) to yield 11 fractions. Previous studies in human pulmonary macrophages from nonsmoking donors and a transformed human macrophage-like cell line, U937, demonstrated that the fraction containing antiviral activity against HSV-1 in macrophage effector cells was the one with an isoelectric point between 1.4 and 2.2 [22]. This fraction contained no detectable IFN-γ activity when tested in a classical interferon assay with human fibroblasts [24] and was not neutralized by monoclonal antibody to IFN-γ [22]. This fraction is therefore referred to as AV-MAF.

A mediator control fraction was prepared in an analogous manner from the supernatant of unstimulated leukocytes. Concanavalin A (10 μg/ml) was added to mediator control supernatant after removal of cells.

Macrophages were activated by exposure to either IFN-γ (500 U/ml) or AV-MAF (50 μl/ml) for 20 hr at 37 C in an atmosphere of 95% room air and 5% CO_2. Before infection, mediator was removed by three washes of the cells in serum-free medium. In previous studies these conditions were the optimal concentration and incubation period to achieve activation of antiviral mechanisms of nonsmoker macrophages in this in vitro system [22]. The concentration of AV-MAF used in these experiments was expressed as μl of the lymphokine-containing fraction concentrated to 1/50th the volume of the original supernatant by vacuum concentration.

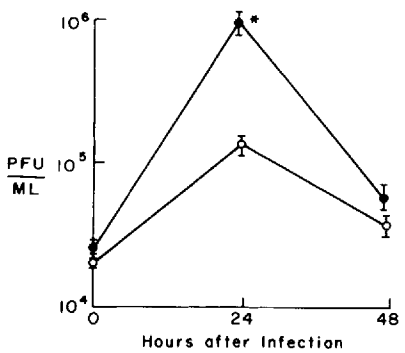

Figure 1. Replication of HSV-1 in untreated pulmonary macrophages from smokers (●) and nonsmokers (○). Virus is quantitated in pfu/ml of macrophage supernatant on the abscissa; time after infection is shown on the ordinate. Data are mean ± SD (bars) values from five experiments. The asterisk indicates $P < .01$ compared with nonsmokers' macrophages.

Control macrophage cultures were exposed to the mediator control fraction (50 μl/ml) in a similar fashion.

Quantitation of cellular protein. The cellular protein concentration was determined in uninfected macrophage cultures grown in serum-free medium after incubation for 3 hr. Nonadherent cells were first removed by gentle washing, and intracellular protein in the remaining adherent cells was released into the culture supernatant by freeze-thawing once at −70 C. The concentration of protein was quantitated by a modification [25] of the Lowry assay, based on the differential A_{595} of Coomassie® brilliant blue G-250 (Bio-Rad, Richmond, Calif) when protein binding occurs.

Statistical analysis. Results were analyzed by a noninpaired Student's t test [26].

Results

Growth of HSV-1 in pulmonary macrophages. Replication of HSV-1 in untreated pulmonary macrophages from smokers and nonsmokers is shown in figure 1. Peak viral growth occurred 24 hr after initiation of infection in cells from both sources; viral growth in smokers' macrophages was 10 times greater than growth in nonsmokers' cells ($P < .01$). This result could not be explained by differences in cell number between smokers' and nonsmokers' cell cultures because the initial number of adherent cells in these cultures before infection was similar and because >80% cell lysis was observed in both smokers' and nonsmokers' cell cultures by 48 hr after infection. At the time of greatest viral replication (24 hr), infectivity of HSV-1 was greater for smokers' cells when corrected for cell count (pfu per cell, 3.39 ± 0.51 and 0.35 ± 0.10 for smokers' and nonsmokers' cell cultures, respectively).

Effect of lymphokine exposure on viral replication. Pretreatment of macrophages with AV-MAF (50 μl/ml) for 20 hr before infection resulted in diminished viral replication in both smokers' and nonsmokers' cells compared with cells exposed to mediator control (figure 2). In cells from nonsmokers, inhibition of viral replication was observed at both 24 and 48 hr after infection. In contrast, smokers' cells exposed to AV-MAF were not capable of totally suppressing viral growth, as evidenced by an increase in the amount of virus recovered during the first 24 hr after infection. Exposure to AV-MAF protected cells from smokers and nonsmokers to a simi-

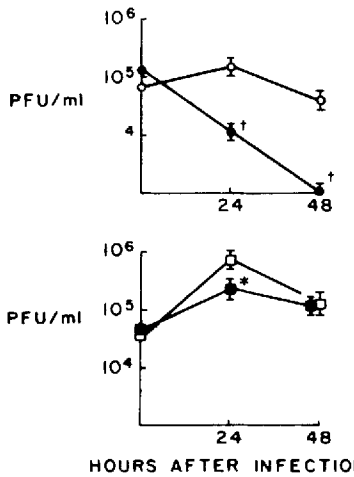

Figure 2. Effect of AV-MAF (closed symbols) versus mediator control (open symbols) on replication of HSV-1 in pulmonary macrophages from nonsmokers (*top; n* = 4) and smokers (*bottom; n* = 6). A dagger indicates $P < .01$ compared with mediator control; an asterisk indicates $P < .05$ compared with mediator control.

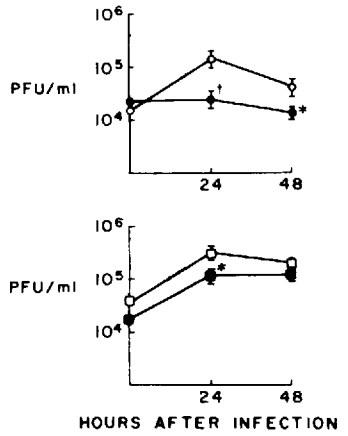

Figure 3. Effect of IFN-γ (closed symbols) versus mediator control (open symbols) on replication of HSV-1 in pulmonary macrophages from nonsmokers (*top; n* = 3) and smokers (*bottom; n* = 4). A dagger indicates $P < .03$ compared with mediator control; an asterisk indicates $P < .05$ compared with mediator control.

lar degree from virus-specific cytolysis. At 48 hr after infection, mean ± SD cytolysis in smokers' cell cultures was 77.7% ± 4% with addition of mediator control and 48.6% ± 1% with addition of AV-MAF, compared with 80.68% ± 1.6% and 48.9% ± 1.8% for nonsmokers' cell cultures, respectively.

Exposure of macrophages to IFN-γ (500 U/ml) diminished viral growth in both smokers' and nonsmokers' cells but only totally inhibited viral replication in nonsmokers' cells (figure 3).

To examine the possibility that the different response of smokers' cells to AV-MAF could be attributable to the use of suboptimal concentrations of this lymphokine to activate cells, we pretreated smokers' macrophages with different amounts of AV-MAF before initiation of infection (table 1). In these studies the maximal antiviral effect was achieved with 50 μl/ml, the amount used in the experiment shown in figure 2. Incubation of cells with 200 μl of AV-MAF/ml resulted in 10%–15% cell death and was not tested further. Exposure of smokers' cells for periods as long as 48 hr or as short as 8 hr to AV-MAF before infection did not result in diminished viral growth compared with cells exposed to this lymphokine for 20 hr (data not shown).

In three experiments nonsmokers' cells were exposed to an increased moi (50–100 virions per cell) to determine whether the different inocula would affect infectivity or lymphokine responses. No increase was observed in the number of virions per cell at 24 hr of infection compared with cells exposed to a lower viral inoculum (20 virions per cell): 0.39 ± 0.18 pfu per cell for the larger inoculum versus 0.35 ± 0.1 as shown earlier for the smaller inoculum. Maximal viral replication and lymphokine-mediated viral inhibition were similarly unaltered (data not shown). Exposure of cells to an inoculum of >100 virions per cell was associated with cytotoxicity within 8 hr after exposure and was not tested further.

Table 1. Effect of AV-MAF concentration on HSV-1 replication in smokers' pulmonary macrophages.

Treatment (μl/ml)	Maximal viral replication ($\times 10^5$)	Maximal virus-specific cytolysis (%)
Mediator control (50)	6.4 ± 1.57	68.36 ± 7.3
AV-MAF		
10	3.8 ± 1.2*	48.06 ± 5†
50	2.6 ± 0.9†	35.83 ± 5.1†
100	2.9 ± 0.2†	31.33 ± 3.3†

NOTE. Maximal viral replication is the highest yield of virus (pfu/ml) found in duplicate macrophage supernatants at either 24 or 48 hr after infection for each experiment. Maximal percentage virus-specific cytolysis is the greatest percentage of cell death attributable to HSV-1 in duplicate cultures at 48 hr after infection in each experiment. Data are mean ± SD values from three experiments.

* $P < .05$ compared with mediator control.

† $P < .01$ compared with mediator control.

Table 2. Adsorption of HSV-1 to pulmonary macrophages.

Source of cells	pfu × 10^5/ 5 × 10^5 cells	pfu × 10^5/μg of protein in 5 × 10^5 cells
Nonsmoker		
No treatment	0.5 ± 0.06	0.2 ± 0.024
Mediator control	0.4 ± 0.07	0.19 ± 0.01
AV-MAF	0.5 ± 0.11	0.2 ± 0.04
Smoker		
No treatment	1.2 ± 0.17*	0.16 ± 0.02
Mediator control	1.16 ± 0.11*	0.15 ± 0.04
AV-MAF	1.0 ± 0.13*	0.187 ± 0.02

NOTE. Adsorption of HSV-1 to macrophages was measured after incubation for 1 hr. Data are mean ± SD values from three experiments.

* $P < .01$ compared with the corresponding nonsmokers' cell culture.

Table 3. Ratio of cell-associated virus to total virus recovered during infection.

Source of cells	Time after infection (hr) 1	24	48
Nonsmoker			
Mediator control	0.85	0.25	0.31
AV-MAF (50 μl/ml)	1.6	0.38	0.35
Smoker			
Mediator control	0.88	0.43	0.38
AV-MAF (50 μl/ml)	2.50	0.28	0.25

NOTE. The ratio of cell-associated HSV-1 to the total amount of virus recovered was determined by separate quantitation of viral pfu in cell lysates (cell-associated virus) and culture supernatants and addition of these values to obtain the total amount of virus for each interval studied. Data are mean values from three experiments.

Cell-associated virus during infection. Because exposure to tobacco smoke alters the ultrastructure of the pulmonary macrophage plasma membrane [27], it is possible that the abnormalities in viral infectivity and lymphokine responses observed in smokers' cells are related to alterations in initial adsorption of virus to the cell surface. Smokers' macrophages exposed or not exposed to lymphokine adsorbed more virus on a per cell basis than did nonsmokers' cells after exposure for 1 hr to HSV-1 (table 2). For determination of whether this finding is the result of altered binding of HSV-1 to the macrophage plasma membrane or is attributable to the larger volume of the smokers' macrophage [28], virus initially adsorbed to cells was quantitated with respect to the amount of protein present in cell lysates of macrophage cultures to account for differences in cell size. There were no differences between smokers' and nonsmokers' cells or lymphokine-exposed and control cells in terms of amount of cell-associated virus per μg of macrophage protein (table 2). Thus, smokers' macrophages adsorbed more HSV-1 at 1 hr after infection on a per cell basis than did nonsmokers' cells, but this difference appears attributable to the larger surface area of smokers' cells.

To examine the possibility that differences between smokers' and nonsmokers' cells are related to an altered capacity of smokers' cells to excrete virus, we measured the ratio of cell-associated virus to the total amount of virus recovered in cell lysates and supernatants. This value was comparable between smokers' and nonsmokers' cells (table 3). It does not appear therefore that increased release of virus into culture medium from smokers' cells accounts for enhanced infectivity of HSV-1 for smokers' cells or for the diminished response of smokers' cells to antiviral lymphokines.

Are abnormal antiviral responses in smokers' cells mediated by a soluble factor? To investigate the possibility of whether smokers' macrophages secrete a substance capable of altering viral infectivity in nonsmokers' cells, we incubated macrophages from nonsmokers with supernatants from unstimulated smokers' macrophages for one day before infection. In three experiments viral replication in nonsmokers' cells was unaffected by prior exposure to supernatant from cultures of smokers' cells. Maximal viral replication was 2.8 ± 1.7 × 10^5 pfu/ml for cells exposed to medium alone and 1.76 ± 1.1 × 10^5 pfu/ml for cells exposed to medium containing smokers' supernatant (50% by volume).

Discussion

These studies demonstrate that cigarette smoking can alter lung cellular defenses against HSV-1 in two distinct ways: (*1*) Smoking enhances infectivity of HSV-1 for macrophages, and (*2*) smoking leads to suboptimal activation of antiviral activity in macrophages by two lymphokines (AV-MAF and IFN-γ). These data describe the interaction of macrophages with a single virus; the relevance of these findings to other respiratory viruses is unclear. However, these observations raise the possibility that abnormal antiviral responses of macrophages may be one pathogenic mechanism underlying the increased incidence and severity of viral infection in the respiratory tract in smokers [1–6, 8–10]. Although exposure to tobacco

smoke can affect other aspects of lung defense, such as mucociliary transport [29], noncellular defenses may be less important than phagocytic cells in eradication of infectious agents from the respiratory tract [30]. For this reason alteration of critical antiviral functions in pulmonary macrophages by cigarette smoke could have a substantial impact on the fate of viruses that reach the lower respiratory tract. Inability of smokers' macrophages to restrict viral replication could lead to increased production of virus within pulmonary airways and parenchyma at an early phase of infection, when the spread of virus cannot be limited by immune factors. Diminished activation of antiviral mechanisms in smokers' macrophages by lymphokines may also interfere with the effectiveness of macrophages at a later stage of infection, after cellular immunity has developed.

In the in vitro system used in these studies to evaluate macrophage antiviral responses, a single viral agent, HSV-1, was tested. This virus is an uncommon cause of respiratory tract infection in immunocompetent humans: In one series, HSV-1 was recovered in 5% of all episodes of community-acquired infections of the lower respiratory tract, compared with an isolation rate of 1.4% in healthy controls [31]. This virus may also be a cause of serious pneumonitis in immunosuppressed individuals [32, 33]. Nevertheless, HSV-1 has been an exceptionally useful agent to probe antiviral responses of macrophages in vitro because it grows rapidly in macrophages, produces cell lysis, and can be inhibited by lymphokine-activated macrophages [22, 34, 35]. One of the lymphokines used in these studies, IFN-γ, has been associated with augmentation of mononuclear phagocyte activity against neoplastic and infectious processes [36–38]. On the other hand, AV-MAF is an unique lymphokine that, like IFN-γ, is capable of activating antiviral activity of macrophages [22] but can be separated from IFN-γ in the supernatant of human peripheral blood leukocytes by preparative isoelectric focusing [22–24]. The suboptimal responses of smokers' pulmonary macrophages to both these lymphokines may have important implications for the effect of smoking on other lymphokine-mediated activities of macrophages, such as killing tumor cells and other intracellular parasites (e.g., *Legionella pneumophila*), because both lung cancer and legionellosis are more common in smokers than nonsmokers [7, 39].

These studies extended the spectrum of functional abnormalities in macrophages that have been attributed to cigarette smoke. Smoking has been shown to depress antibacterial activity of pulmonary macrophages in vitro [40–42] and inhibit phagolysosome fusion [43], a crucial mechanism for eradication of certain intracellular microbes. Only one previous study assessed the impact of smoking on macrophage responses to lymphokines. Human pulmonary macrophages from smokers had diminished responses to macrophage migration-inhibitory factor [44]. These functional alterations may be based on metabolic or structural changes in macrophages produced by exposure to tobacco smoke. For instance, macrophage levels of the energy-generating enzymes adenosine triphosphatase [45] and glucose-3-phosphate dehydrogenase [46] are diminished in smokers' cells and may be responsible for lowered rates of phagocytosis observed in smokers' macrophages. Similarly, smoking-related ultrastuctural changes, consisting of increased blebs and lamellipodia, in the macrophage plasma membrane have been implicated in altered pinocytosis and endocytosis [27].

Although in the present studies we did not elucidate the mechanism(s) responsible for increased infectivity and diminished lymphokine response in smokers' macrophages, these alterations could not be explained by increased release of intracellular virus into culture medium during infection (table 3) or by differences between the optimal concentration of lymphokine needed to produce an antiviral state in smokers' and nonsmokers' cells (table 1). Moreover, macrophage abnormalities associated with smoking do not appear to be the result of exposure of cells to a soluble factor secreted by smokers' macrophages because incubation of nonsmokers' cells with supernatant from smokers' cell cultures did not alter the infectivity of HSV-1 for cells treated in this manner.

Altered antiviral responses of smokers' macrophages may be due in part to enhanced initial adsorption of HSV-1 (table 2). Because the amount of virus adsorbed to smokers' and nonsmokers' cells is similar when corrected for intracellular protein concentration (table 2), increased uptake of HSV-1 by smoker's cells appears to be related to a larger surface area available for attachment of virus. This conclusion is in contrast to a previous study that found only a small size difference in these cell populations on the basis of direct measurement of cell diameter [28]. This method of assessing cell size could underestimate true cell volume compared with

measurements of cellular protein concentration. By the latter method smokers' cells appeared nearly twice as large as nonsmokers' cells. On a per cell basis, smokers' macrophages are exposed to more than twice the amount of adsorbed virus as are nonsmokers' cells (table 2). It may be, therefore, that increased infectivity of HSV-1 for smokers' cells results from greater initial adsorption of virus, diminished ability of the extra protein in smokers' cells to limit viral replication, or both.

Thus, smoking appears to depress the intrinsic antiviral activity of macrophages against HSV-1 and diminish the augmentation of this activity by lymphokines. Further studies are needed to clarify the relevance of these observations for other respiratory viruses and other lymphokine-directed macrophage functions. The importance of smoking in acquired antiviral and immune deficiencies of macrophages remains speculative.

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