Age-Dependent Resistance of Human Alveolar Macrophages to Herpes Simplex Virus

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Studies in mice demonstrate an age-dependent susceptibility to disseminated herpesvirus infection which is mediated, at least in part, by a defect in macrophage antiviral function. We examined the growth of herpes simplex virus within human alveolar macrophages obtained by bronchopulmonary lavage from neonates, adults with a variety of immunosuppressive disorders, and healthy adult volunteers. At 24 h postinfection, mean viral titers in neonatal macrophages increased 19-fold over adsorbed virus levels, a highly significant increase when compared to either immunosuppressed or normal adult macrophages ($P < 0.0005$). These findings indicate that human macrophages, like those of mice, exhibit age-dependent permissiveness for the replication of herpes simplex virus. This permissiveness may at least partially account for the clinical observation that human newborns are highly susceptible to disseminated herpes simplex virus infections, whereas adults are not.

Macrophage response is a major determinant of host susceptibility to a number of viruses (2, 9, 11, 12, 21). In laboratory animals, macrophage antiviral activity has been shown to be age dependent (11, 12, 21). Suckling mice are more susceptible than adults to fatal, disseminated herpes simplex virus (HSV) infections; their macrophages release more infectious virus and more readily spread infection to adjacent uninfected cells (11, 12). Human newborns are also more susceptible than adults to disseminated HSV infection (17, 19). To determine whether human newborn macrophages share the "permissiveness" of suckling mouse macrophages, we have compared the replication of HSV in newborn and adult human alveolar macrophages (AM).

MATERIALS AND METHODS

Tissue culture cells and media. Human embryonic kidney cells, WI-38 human fetal diploid lung fibroblasts, and the BSC-1 continuous monkey kidney cell line were all obtained commercially (Microbiological Associates, Bethesda, Md.) and grown in Eagle minimal essential medium containing 8% heat-inactivated fetal bovine serum and antibiotics (gentamicin, 50 $\mu\text{g}/\text{ml}$, and amphotericin B, 1.25 $\mu\text{g}/\text{ml}$). Human AM were maintained in modified McCoy 5a medium (M-McCoy) containing 20% fetal bovine serum and antibiotics. Hanks balanced salt solution (HBSS), con-

taining antibiotics and 2% fetal bovine serum, was used throughout as a diluent.

Virus. The McIntyre strain of HSV type 1 (HSV-1) was kindly provided by Douglas Ehresman (U.S. Naval Research Laboratories, Oakland, Calif.). Stock virus (5×10^7 plaque-forming units per ml) was grown in human embryonic kidney cells, liberated by sonication at 10,000 Hz for 30 s, clarified by centrifugation at $1,000 \times g$ for 20 min, and frozen at -70°C until used.

Macrophage donors. AM were obtained by bronchopulmonary lavage (see below) from three groups of donors as follows. (i) The first group included nine hospitalized adults with a variety of immunosuppressive disorders (lymphoma, leukemia, sarcoidosis, metastatic carcinoma) who underwent diagnostic bronchopulmonary lavage as part of the workup of a pulmonary infiltrate. (ii) The second group was comprised of seven healthy adult volunteers from whom informed consent was obtained. Cigarette smokers were chosen since they consistently yield greater numbers of AM than do nonsmokers (22). Macrophages were also obtained from two volunteer nonsmokers (data not shown). Despite the very low yield of cells, their AM behaved identically to those obtained from smokers when infected with HSV. (iii) The third group included three neonates (less than 2 weeks old) who died in the intensive care nursery and who were lavaged post mortem with informed parental consent. The underlying illnesses in these three infants were sepsis, neonatal respiratory distress syndrome, and intracranial hemorrhage, respectively.

Serological studies were not routinely performed on AM donors. However, HSV complement-fixing antibody titers were available from four of the adult volunteers (two had titers $< 1:8$; two had titers of 1:16 and 1:64, respectively). No quantitative or qualitative

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differences were observed among them when their AM were infected with HSV (data not shown).

Macrophages. Human adult AM were obtained by segmental bronchopulmonary lavage under fluoroscopic monitoring as previously described (7). Neonatal AM were obtained from cadavers within 1.5 h of death. Briefly, a 2.5-mm Portex endotracheal tube was inserted, and through it a sterile no. 5 French polyethylene catheter was passed blindly to the level of the carina. Lavage was performed 8 to 10 times with 10-ml volumes of HBSS, and all effluent fluids were pooled. Adult and neonatal AM samples were transported to the laboratory on ice, pelleted by centrifugation at $500 \times g$ for 10 min, and suspended in M-McCoy medium. Samples of all donor macrophages were inoculated into tubes of WI-38 and human embryonic kidney cells and were found on culture to be free of endogenous viral contamination. Cells were counted in a hemacytometer, and viability was determined by trypan blue dye exclusion. Adult lavages yielded from 1×10^7 to 6×10^7 viable AM, while neonatal lavages generally yielded between 5×10^5 and 10×10^5 viable AM. Cell concentrations were adjusted to approximately 5×10^5 viable AM per ml for adults and 5×10^4 viable AM per ml for neonates. Volumes of 1 ml of the respective AM dilutions were seeded into 16-mm plastic wells of Multiwell Tissue Culture Plates (Falcon, Oxnard, Calif.) and were allowed to acclimate for 24 h at 37°C in a high-humidity atmosphere containing 5% CO_2 . After acclimation, medium was aspirated, and the cells were washed twice with HBSS. Giemsa-stained adherent cells from all donors were morphologically similar. On the basis of plastic adherence and morphological appearance, the remaining cells were considered an essentially pure culture of AM. Viability of adherent cells from all donors was consistently in the range of 93 to 97%.

Viral growth curves. Washed AM were challenged with 0.5 ml of HSV stock virus diluted in HBSS to obtain a multiplicity of infection of between 10 and 40. A high multiplicity was employed to assure infection of greater than 90% of AM. Virus was allowed to adsorb for 1 h at 22°C , and then the inocula were aspirated, and the cells were washed three times with HBSS, covered with 1 ml of M-McCoy medium, and incubated at 37°C in a high-humidity 5% CO_2 atmosphere. At timed intervals the medium was aspirated from infected wells, 1 ml of HBSS was added, cells were scraped into this solution, and yields of replicate wells were pooled with their respective volumes of M-McCoy medium and frozen at -70°C until viral assay was performed. Similarly, cell-free wells containing 10^5 plaque-forming units of HSV-1 in 1 ml of M-McCoy medium were incubated, harvested at intervals, frozen, and assayed as described below to provide a decay curve of viral infectivity in a cell-free environment.

Virus assay. HSV plaque assays were performed by a modification of a method previously described (5). Samples of virus-infected AM were thawed and sonicated for 30 s at 10,000 Hz to release intracellular virus. Serial fivefold dilutions of HSV-1 stock virus or sonicates of AM were made in HBSS and inoculated in triplicate in 0.2-ml volumes onto monolayers of BSC-1 cells grown in 16-mm plastic wells. After allowing 1 h at 22°C for viral adsorption and an additional 1 h at 37°C to facilitate viral penetration, the inocula

were removed, and the monolayers were overlaid with 1 ml of Eagle minimal essential medium containing antibiotics, 2% fetal bovine serum, and 0.4% pooled human immune serum globulin (Hyland, Costa Mesa, Calif.). This overlay permits plaque formation by direct cell-to-cell spread of virus, but prevents secondary plaque formation (5). After 48 to 72 h of incubation at 37°C in a high-humidity 5% CO_2 atmosphere, the medium was removed, monolayers were fixed for 10 min in 10% Formalin, and unstained plaques were counted macroscopically.

Statistical evaluation was performed by analysis of variance utilizing Scheffé's method of multiple comparisons (10).

RESULTS

Growth of HSV in AM. HSV-1 growth curves in AM from three neonates and from seven normal and nine immunosuppressed adults are depicted in Fig. 1. A decline in viral titer occurred during the first 12 h of incubation and was more pronounced in adult than in neonatal AM ($P < 0.01$). Thereafter, an increase in infectious virus titer was observed in all groups. At 24 h the viral titer in AM from immunosuppressed adults was 46% of the adsorbed (2-h) titer, whereas the titer in normal adult AM had increased only 1.8-fold over the 2-h sample. The

FIG. 1. Single-cycle growth curves of HSV-1 in human AM. Mean viral titers ($\pm$ standard error of the mean) are expressed as a percentage of the adsorbed (2-h) virus titer for macrophages and permissive BSC-1 cells, and as a percentage of the input virus titer for the cell-free control.

difference between the two adult groups was not statistically significant. In contrast, the 24-h virus titer in neonatal AM increased 19-fold over adsorbed virus levels, a highly significant difference when compared with either adult group ($P < 0.0005$, Table 1). Over the same period of time, viral titers in macrophage-free control wells steadily declined to 2.5% of input levels (Fig. 1). Continued incubation of infected AM for up to 72 h resulted in no further change in viral titer in any of the groups studied (data not shown).

Effect of macrophage concentration on viral growth. Because neonatal lavages yielded far fewer AM than adult lavages, neonatal test wells were routinely seeded with approximately one-tenth the number of AM that adult wells received. To determine whether AM concentration affected the outcome of viral growth curves, separate studies were performed on adult AM in high and low concentrations (3.5×10^5 and 4.3×10^4 AM per well, respectively) infected with the same viral multiplicity per cell (10:1). Results from a representative sample are presented in Fig. 2. Viral titers, expressed as a percentage of the adsorbed (2-h) virus titer, were essentially identical for both high and low AM concentrations throughout the incubation period.

DISCUSSION

Age-dependent resistance of mice to HSV infection was first observed by Andervont (1) and subsequently confirmed by others (11, 12, 14). Specifically, intraperitoneal inoculation of suckling mice with HSV leads to a rapid spread of virus to viscera, followed by encephalitis and death, whereas similar inoculations into adult mice are not followed by viral dissemination and death.

Work by several investigators (2, 9, 15) suggests that macrophage function is an important determinant in the outcome of certain viral infections. Johnson (12) demonstrated that HSV-infected suckling mouse macrophages readily transmitted virus to surrounding uninfected cells, but adult mouse macrophages did not.

TABLE 1. Percent of adsorbed HSV-1 recovered from neonatal and adult AM

AM source	No. of patients	Incubation time (mean % $\pm$ SEM ^a)	
		12 h	24 h
Neonates	3	$\{96.7 \pm 21.1\}^c$	$\{1,860 \pm 185\}^c$
Normal adults	7	$\{32.8 \pm 11.6\}^d$	$\{176.1 \pm 74.5\}^d$
Immunosuppressed adults	9	$\{27.3 \pm 8.7\}^d$	$\{45.7 \pm 15.9\}^d$

^a SEM, Standard error of the mean.
^b $P < 0.01$.
^c $P < 0.05$.
^d Not statistically significant.
^e $P < 0.0005$.

FIG. 2. Effect of macrophage concentration on viral growth. AM from a single adult donor at high (3.5×10^5 cells per well) and low (4.3×10^4 cells per well) concentrations were infected at a multiplicity of 10.

Hirsch et al. (11) extended these observations by demonstrating that HSV-infected suckling mouse peritoneal macrophages formed from 7 to 47 times as many infectious centers in vitro as did adult macrophages. More importantly, they confirmed the central role of macrophages in controlling clinical infection by showing that transfer of large numbers of proteose-peptone-stimulated adult macrophages to syngeneic suckling mice subsequently infected with HSV reduced mortality from 85 to 26%. Such protection was not conferred by transfer of: (i) smaller numbers of stimulated macrophages; (ii) large numbers of unstimulated macrophages; (iii) proteose-peptone alone; or (iv) large numbers of adult peritoneal lymphocytes.

Studies of the mechanism by which mouse macrophages limit HSV replication (24) indicate that the macrophage adsorbs virus and produces viral deoxyribonucleic acid and proteins but restricts viral assembly. This results in abortive infection, characterized by the production of viral capsids with low-density cores and the release of strikingly few complete infectious virions.

As in laboratory animals, the susceptibility of humans to disseminated HSV infection appears to be age dependent. Whereas widespread dissemination of HSV is decidedly uncommon in older children and adults (17), approximately two thirds of infants with neonatal HSV infection exhibit visceral dissemination (19). This occurs despite the presence of circulating antibody in maternal and newborn blood (18); thus, a non-antibody-mediated defect in antiviral defense is presumed to be present.

Our experiments demonstrate that HSV replication is significantly greater (10-fold, $P < 0.0005$) in newborn than in adult human AM. AM were chosen for study because relatively large numbers can be easily obtained, and because involvement of the lung is common in

disseminated infection of newborns (18, 25, 27) but rare in HSV infection of adults (3, 4, 13, 20).

Interestingly, AM from immunocompromised patients were not impaired in their ability to limit HSV replication. These patients had many different underlying diseases and therapies associated with immunosuppression, yet in no instance did HSV-1 replicate in their macrophages to the same magnitude as in newborn cells. Although an increased frequency of localized and often severe HSV infection has been reported in patients with hematological malignancies (16) and in immunosuppressed cardiac transplant recipients (23), visceral dissemination was rarely observed. The role of the macrophage in protecting these patients from disseminated HSV infection is speculative. However, Golde and co-workers (8) have noted that bronchopulmonary lavage of leukopenic leukemic patients yields AM that appear normal in both number and function.

It is also noteworthy that macrophage antiviral function appears to be virus specific as well as age dependent. Mouse macrophages develop resistance with age to infection by Wesselsbron virus, but remain fully susceptible to Germiston virus infection at all ages (21). Similarly, we have previously reported that adult human alveolar macrophages are permissive for the replication of cytomegalovirus while markedly restricting the growth of HSV (6).

The present investigation offers only indirect evidence for an age-dependent protective role of macrophages against disseminated HSV infections in humans; however, it corroborates *in vitro* findings of earlier studies (2, 9, 11, 12) that clearly demonstrate macrophage-mediated antiviral protection in laboratory animals.

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