INFECTION AND IMMUNITY, Feb. 1979, p. 432-437
0019-9567/79/02-0432/06$02.00/0

Vol. 23, No. 2

Replication of Herpes Simplex Virus Type 1 in Macrophages from Resistant and Susceptible Mice

CARLOS LOPEZ* AND GREGORY DUDAS

Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Received for publication 20 November 1978

Studies were carried out to determine whether the in vitro capacity of adherent peritoneal cells to replicate herpes simplex virus type 1 (HSV-1) might correlate with the in vivo susceptibility of mice genetically resistant, moderately susceptible, or very susceptible to HSV-1 infection. Unstimulated and proteose peptone-stimulated monolayers restricted viral replication when infected immediately, but replicated HSV-1 when infected after 3 to 7 days of culture. Macrophages from resistant C57Bl/6 mice restricted HSV-1 replication significantly better than cells from susceptible mice. This function did not segregate with resistance, since macrophages from resistant F_1 mice failed to restrict HSV-1 replication. Induction of peritoneal exudate cells with thioglycolate yielded cells capable of replicating HSV-1 when infected immediately after plating and after 4 days of culture.

Using inbred strains of adult mice, we have demonstrated mice genetically resistant, moderately susceptible, and very susceptible to an intraperitoneal inoculation with a virulent strain of herpes simplex virus type 1 (HSV-1) (13). Although all seven HSV-1 strains tested to date have yielded the same pattern of resistance in the three prototype mouse strains (C57Bl/6 = resistant; BALB/c = moderately susceptible; and A/J = very susceptible), the various strains of virus were shown to demonstrate weak to high virulence (13). When inoculated intracerebrally, all three strains of mice were found to be very susceptible, indicating that if virus gets to the target cells it is capable of replicating and killing in all three strains. Earlier observations (C. Lopez, *in Oncogenesis and Herpesviruses*, in press) as well as more recent findings indicate that resistance is immunological in nature. Thus resistance in mice can be abrogated by treatment of resistant mice by agents which impair either macrophage or lymphocyte (probably T-cell) function. In addition, reconstitution of lethally irradiated susceptible mice with bone marrow cells from resistant F_1 mice yielded mice resistant to HSV-1 infection (Lopez, in press).

The early experiments of Johnson (12) demonstrated that the in vitro replication of HSV in peritoneal macrophages from suckling mice could be correlated with their susceptibility to intracerebral inoculation of the virus. Macrophages from adult mice restricted the replication of the virus in vitro, and that was associated with in vivo resistance to HSV. These observations were followed by studies of Zisman et al. (23), who demonstrated that impairing macrophage function in adult mice resulted in an increased susceptibility to an intraperitoneal inoculation with HSV. Hirsch et al. (11) additionally demonstrated the protective effect of adult macrophages in suckling mice. These studies and the more recent in vitro studies of Stevens and Cook (21) indicate an important role for the macrophage, and in particular its capacity to restrict the replication of HSV, in resistance to the virus infection.

Because of the observations of Johnson (12) and the follow-up studies of Zisman et al. (23) and Hirsch et al. (11), it became important to determine whether in vitro restriction of HSV-1 replication by macrophages from resistant versus susceptible strains of mice might account for in vivo resistance. The studies described herein indicate that the in vitro capacity of peritoneal macrophages to restrict HSV-1 replication depends on the inducing agent used to obtain the cells and the period of time in culture prior to their infection. We conclude that this function does not correlate with genetic resistance.

MATERIALS AND METHODS

Mice. C57Bl/6, BALB/c, A/J, (C57Bl/6 × BALB/c)F_1, and (C57Bl/6 × A/J)F_1 male mice were obtained from Jackson Laboratory, Bar Harbor, Maine. All mice were 3 to 4 months old when used.

Media. All cells were propagated as monolayers on plastic in Dulbecco-modified Eagle minimal essential medium (DME) supplemented with 10% (prescreened) heat-inactivated fetal calf serum, fresh glutamine, and antibiotics (penicillin and streptomycin) (GIBCO, Grand Island, N.Y.).

Cells. Vero cells were obtained from the American Type Culture Collection and were used for propaga-

tion and plaque assay of all HSV preparations.

Macrophages were obtained from either unstimulated mice or mice stimulated 4 days earlier with an intraperitoneal inoculation of 1.5 ml of 10% proteose peptone (Difco, Detroit, Mich.) in phosphate-buffered saline or 1.5 ml of 10% Brewer thioglycolate (Difco, Detroit, Mich.) in phosphate-buffered saline. Each 10% solution was sterilized by autoclaving for 15 min before use. Macrophages were obtained from the peritoneal cavity by standard methods (8). In earlier experiments, macrophages were enumerated by determining the number of latex-ingesting cells (17) in a hemacytometer. Since later experiments in which macrophages were enumerated with the aid of crystal violet in acetic acid (2) yielded identical results faster and with more reproducibility, the latter was used throughout most of this study. Cells with a generous cytoplasm and reniform nuclei were considered macrophages.

In most experiments, 10^6 macrophages were plated in each well of a Linbro FB-16-24-TC plate (Linbro Chemical Co., Inc., New Haven, Conn.) in 1 ml of medium. After a 2-h incubation period in a humidified CO_2 incubator at 37°C, the monolayers were washed three times with warm (37°C), serum-free DME. Peritoneal cells to be treated with anti-thy 1.2 serum and complement were suspended in DME at a concentration of 3×10^6 macrophages per 3 ml. Equal portions of AKR mouse antiserum to C3H thy 1.2 (19) at a dilution of 1:32 and rabbit complement diluted 1:15 were added to the cell suspension. These cells were then plated and washed as described above. Control cells were treated with fetal calf serum and complement.

Macrophages were infected with virus either immediately after the last wash or after the cells had been incubated in media for 3 to 7 days. After the additional incubation, monolayers were washed three more times before infection.

The AKR anti-C3H thy 1.2 was prepared as described by Reif and Allen (19). Although slightly fewer cells adhered to the plastic surface in the presence of the anti-thy 1.2 antiserum plus complement, it was not obviously cytotoxic for C57Bl/6 or BALB/c adherent peritoneal cells during or after adsorption. By comparison, an anti-H-2b antiserum plus complement was cytotoxic for C57Bl/6 adherent cells after adsorption and precluded adherence.

Virus. HSV-1 strain 2931 was isolated from a patient with herpes labialis and was used in the study of genetic resistance of mice to HSV-1 (13). HSV-1 strain F was a gift from B. Roizman. As described earlier, strain 2931 is the most virulent strain of HSV-1 tested in the mouse model to date (13). Strain F, on the other hand, is relatively avirulent in mice after intraperitoneal inoculation; the 50% lethal dose for A/J strain mice was 10^4 plaque-forming units. By comparison, the 50% lethal dose of HSV-1 strain 2931 in A/J mice was $10^{1.3}$ plaque-forming units (13).

Medium was removed from macrophage monolayers before infection with virus. Cells were inoculated with virus in a volume of 0.3 ml. After a 2-h adsorption period in a humidified CO_2 incubator (37°C), the monolayers were washed three times with DME and overlaid with 1 ml of DME. At the specific times postinoculation, cells were scraped into the medium with a rubber policeman, triturated, and transferred to plastic tubes for storage (−70°C). Virus was released from cells by rapid freezing and thawing (3×). After centrifugation at $300 \times g$ to remove cellular debris, virus was titrated on Vero cell monolayers in Micro Test II plates (Falcon Plastics, Oxnard, Calif.) as described earlier (14).

All comparative studies were carried out simultaneously and were usually done in duplicate. Statistical evaluation was by Student's t test (1).

RESULTS

Macrophage monolayers established from unstimulated or proteose peptone-stimulated C57Bl/6, BALB/c, and A/J mice restricted the replication of HSV-1 when inoculated immediately after the establishment of the monolayers. Figure 1A demonstrates the restriction of replication of HSV-1 (F strain) by proteose peptone-induced peritoneal macrophages. Results similar to these were obtained with unstimulated macrophages (data not presented). These findings were consistent (six experiments) and always demonstrated greater inactivation of virus by these macrophage monolayers than by heat-inactivation of the virus at 37°C. Peritoneal macrophages from C57Bl/6 mice consistently restricted the replication of HSV-1 more effectively than the cells obtained from BALB/c or A/J mice. However, these differences were statistically significant in only one of six experiments ($P < 0.01$). Results similar to those described above were obtained with HSV-1 strain 2931 (data not presented), demonstrating similar results with a virulent strain of virus. Additional experiments carried out using different multiplicities of infection demonstrated growth curves parallel to those described above (data not presented).

Maintaining the macrophage monolayers in culture for 4 days before infecting with HSV-1 yielded monolayers clearly capable of replicating the virus (Fig. 1B). Again, HSV-1 (2931) replicated as well as HSV-1 (F strain), indicating no difference in the capacity to replicate virulent versus avirulent strains of HSV-1. When infected 4 days after plating, macrophage monolayers from C57Bl/6 mice consistently replicated HSV-1 significantly less well than macrophages from A/J strain and BALB/c mice ($P < 0.01$, 1 day postinoculation). Maintaining C57Bl/6 macrophages in culture for 7 days, however, demonstrated that they were also capable of replicating HSV-1 after culturing for a sufficiently long period of time (data not presented). Monolayers inoculated with different multiplicities of infection (0.01 to 10) after 4 days of culture demonstrated similar growth curves (data not pre-

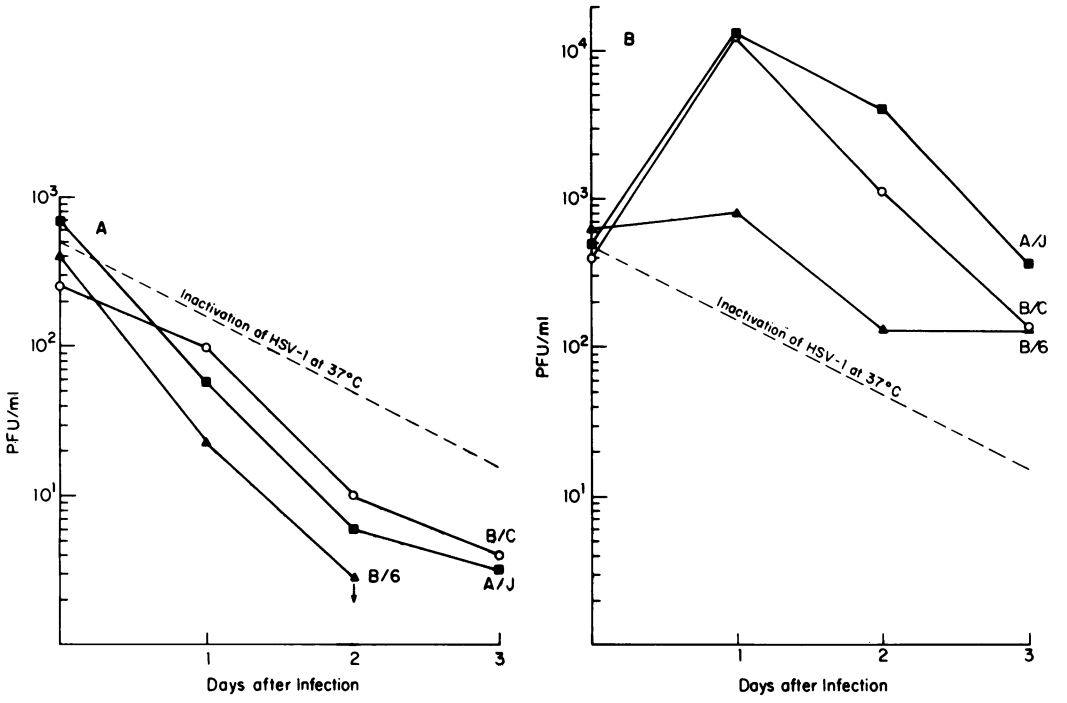

FIG. 1. *Replication of HSV-1 in monolayers of adherent peritoneal cells stimulated with proteose peptone. After adsorption to plastic, monolayers were washed thoroughly and infected immediately or cultured in DME with 10% fetal calf serum for 4 days prior to a second thorough washing and infection with HSV-1. (A) C57Bl/6 (B/6), A/J, and BALB/c (B/c) adherent cell monolayers were infected at a multiplicity of infection of 1 plaque-forming unit per cell immediately after plating cells. (B) Adherent cell monolayers were cultured for 4 days prior to infection. B/c and A/J mouse adherent cell monolayers replicated HSV-1 significantly better than B/6 monolayers at 1 day postinfection ($P < 0.01$) and at 2 days postinfection ($P < 0.05$).*

sented); the only difference was a slight delay in peak viral titer in cells inoculated with low multiplicities of infection. The amount of virus adsorbed by macrophage monolayers was always proportional to the amount of virus used for inoculation.

Treatment of peritoneal cells with AKR anti-C3H thy 1.2 and complement during the adsorption period appeared to diminish slightly the number of cells that adhered to the plastic surface. Treated monolayers, however, demonstrated virus growth curves similar to those of untreated monolayers (Fig. 2). The anti-thy 1.2-treated monolayers adsorbed the same amount of virus and replicated the virus as well as untreated monolayers.

Macrophage monolayers from C57Bl/6 mice consistently replicated HSV-1 less well than the macrophages from either the BALB/c or A/J mice when inoculated after 4 days in culture. Since C57Bl/6 mice are resistant to HSV-1 infection in vivo, this capacity of their macrophages to restrict HSV-1 replication might be an important aspect of that resistance. To determine whether this function segregated with resistance, (C57Bl/6 × BALB/c)F_1 and (C57Bl/6 × A/J)F_1 mice were used to establish macrophage monolayers, and these monolayers were tested for their capacity to replicate HSV-1 when inoculated 4 days after plating. Since F_1 mice are resistant to HSV-1 infection, restriction of viral replication would indicate that this in vitro function segregated with in vivo resistance. Macrophage monolayers from both F_1 strains, inoculated after 4 days in culture, showed a capacity to replicate HSV-1 more like that of the susceptible than the resistant parent. At 1 day postinoculation (Fig. 3A), macrophages from F_1 mice replicated the virus more like the susceptible parent and significantly better than the resistant C57Bl/6 parent ($P < 0.001$). Similar experiments (Fig. 3B) with unstimulated macrophages infected immediately after plating showed that cells from resistant F_1 mice were unable to restrict viral replication as effectively as cells from the resistant parent ($P < 0.01$). Therefore, the capacity of macrophages from C57Bl/6 mice to restrict HSV-1 replication im-

mediately after plating or 4 days later does not segregate with resistance, since cells from resistant F_1 mice did not exhibit this trait.

Thioglycolate-stimulated macrophage monolayers were also inoculated with HSV-1 immediately after plating and 4 days later. Unlike unstimulated or proteose peptone-stimulated macrophages, thioglycolate-stimulated macrophage monolayers replicated HSV-1 immediately after plating and, to an even higher degree, after 4 days in culture (Fig. 4). As with the unstimulated and proteose peptone-stimulated C57Bl/6 macrophages, inoculation of thioglycolate-stimulated C57Bl/6 macrophages demonstrated less viral replication than BALB/c or A/J strain mouse macrophages. The restriction, however, did not segregate with resistance, since the macrophages from both F_1 strains replicated the virus significantly better [$P < 0.001$ for (C57Bl/6 × A/J)F_1 and $P < 0.01$ for (C57Bl/6 × BALB/c)F_1] than the cells of the resistant C57Bl/6 parent (Fig. 4).

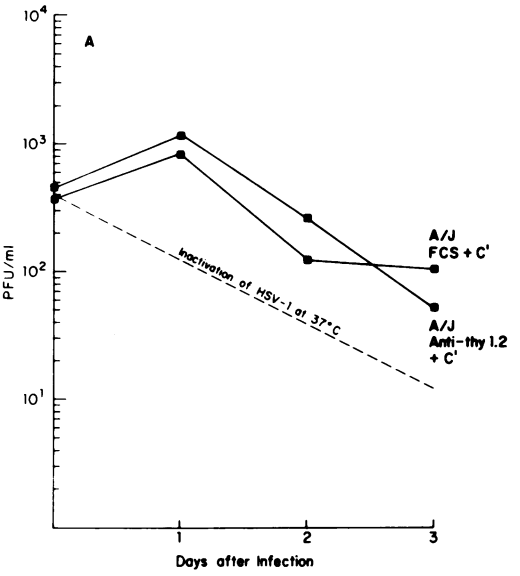

FIG. 2. *Effect of treatment of adherent peritoneal cells with AKR anti-C3H thy 1.2 and complement on the capacity of the cells to replicate HSV-1 strain 2931. Replication of HSV-1 in monolayers treated during adsorption period and infected after culturing for 3 days is not significantly different in monolayers treated with anti-thy 1.2 and complement than in control cultures treated with calf serum and complement.*

DISCUSSION

These studies were initiated to determine whether in vitro replication of HSV-1 by macrophages from resistant, moderately susceptible, and susceptible strains of mice correlated with in vivo resistance. Although macrophages from resistant C57Bl/6 mice restricted HSV-1 replication better than macrophages from moderately susceptible BALB/c and very susceptible A/J strain mice, this capacity did not correlate with genetic resistance, since macrophages from resistant (C57Bl/6 × A/J)F_1 and (C57Bl/6 × BALB/c)F_1 strain mice failed to restrict HSV-1 replication. Thus, although the age-related susceptibility of mice to HSV-1 infection appears to depend on the capacity of the host's macrophages to restrict viral replication, a similar mechanism does not appear to play a significant role in genetic resistance of adult mice.

Our study indicates that the state of activation of the macrophages appears to be an important determinant of their capacity to replicate HSV-1. Proteose peptone and thioglycolate are two commonly used inducers of stimulated (inflammatory) macrophages (5). When compared to unstimulated macrophages, proteose peptone- and thioglycolate-induced macrophages spread faster on glass (3, 4), demonstrate increased pinocytosis (10), show an ability to ingest complement-coated sheep erythrocytes (10), display elevated rates of synthesis and secretion of a variety of neutral proteases (4), and have diminished or absent levels of 5'-nucleotidase activity (9). Usually, thioglycolate stimulation demonstrated more pronounced changes than proteose peptone stimulation. Our study demonstrated that thioglycolate-stimulated macrophage monolayers replicated HSV-1 much better than unstimulated or proteose peptone-stimulated cells. Additionally, when unstimulated or proteose peptone-stimulated macrophages were cultured for 3 to 7 days prior to inoculation with HSV-1, the monolayers acquired the capacity to replicate HSV-1 (or lost their capacity to restrict HSV-1 replication). This was found to be the case even though similar culturing of resident (unstimulated) macrophages has been associated with their acquiring some of the characteristics of stimulated cells (4). Our studies, thus, suggest that stimulation of macrophages yields cells more capable of replicating HSV-1.

An important question is raised by this study: what is the fundamental difference between macrophage monolayers capable of replicating HSV-1 and those which restrict replication? One possibility is that stimulated macrophages have gained specific precursors required for replication of the virus (6). These cells could, on the other hand, lose an HSV-1-suppressive function found in resident macrophages. For example, nonphagocytic adherent cells (NPAC) may exhibit an antiviral activity as well as the tumor-

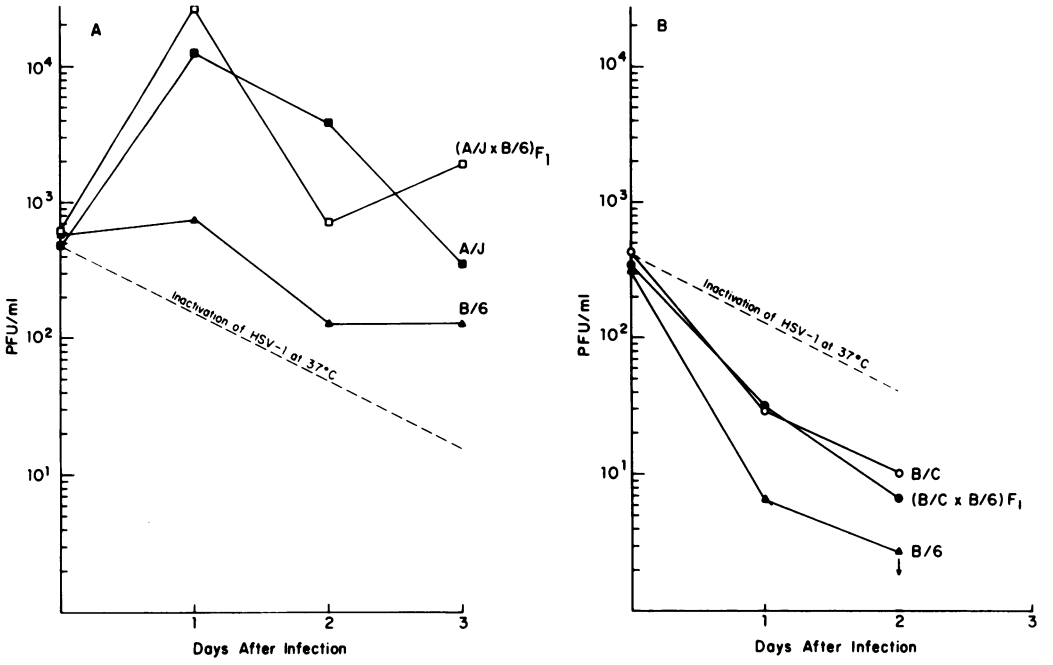

FIG. 3. *(A) Replication of HSV-1 strain 2931 in proteose peptone-induced adherent cell monolayers established from C57Bl/6 (B/6), A/J, and (B/6 × A/J)F₁ mice inoculated after culturing for 4 days. HSV-1 replicated in cells from resistant F₁ mice significantly better than in the cells from resistant C57Bl/6 mice [P < 0.001 for (B/6 × A/J)F₁]. Similar experiments with B/6, BALB/c (B/c), and (B/6 × B/c)F₁ mice demonstrated similar findings. (B) Replication of HSV-1 strain 2931 in unstimulated adherent cell monolayers established from B/6, B/c, and (B/6 × B/c)F₁ mice inoculated immediately after plating cells. HSV-1 was restricted by cells from F₁ mice as well as from susceptible (B/c) mice and not as well as cells from resistant (B/6) mice (P < 0.01).*

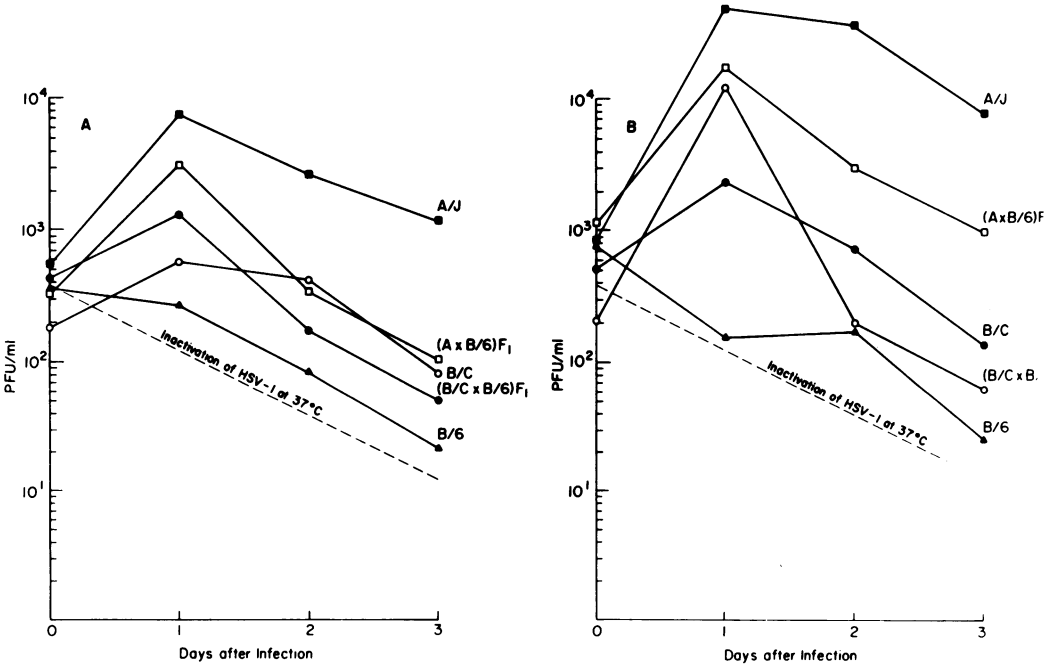

FIG. 4. *Replication of HSV-1 strain 2931 in thioglycolate-induced adherent peritoneal cell monolayers from C57Bl/6 (B/6), A/J, BALB/c (B/c), (B/6 × A/J)F₁, and (B/6 × B/c)F₁ mice. HSV-1 replicated in all cells infected immediately after plating (A) and, even to a greater extent, in monolayers cultured for 4 days prior to infection (B). Although cells from C57Bl/6 mice restricted viral replication better than cells from A/J and BALB/c mice, this property did not segregate with resistance, since cells from resistant F₁ mice did not restrict viral replication.*

inhibitory function recently described for them (18). Nathan et al. (18) found that about 6% of the unstimulated adherent peritoneal cells are NPAC, whereas less than 2% of thioglycolate-induced cells are NPAC. Furthermore, culture of adherent peritoneal cells led to the steady loss of NPAC from the monolayers, and nearly all NPAC were dead after 5 days of culture (18). Because of the properties of the NPAC, which indicate that they are probably absent or in very low concentrations in adherent peritoneal cultures that replicate HSV-1, it is possible that these cells play an important role in the induction of macrophage monolayers capable of restricting HSV-1 replication. Studies are in progress to determine whether the differences demonstrated in our study can be attributed to the concentration of NPAC in the adherent cell monolayers.

Although activation of macrophages by inflammatory stimuli leads to cells with many of the morphological and biochemical characteristics of macrophages activated immunologically (3, 7, 15, 16, 22), the former clearly do not demonstrate the bactericidal capability of the latter (15). It is thus possible that, in the in vivo situation, antigenic stimulation is required for the activation of "virucidal" capacity of macrophages. Infection with bacille Calmette-Guérin (BCG) has been shown to activate macrophages that are nonspecifically bactericidal (16) and to enhance markedly the resistance to HSV-2 in vivo (20). We have found that BCG infection augments resistance to HSV-1 and that macrophages from BCG-infected mice restrict HSV-1 replication even when stimulated with thioglycolate or after 4 days of culturing (Lopez and Dudas, manuscript in preparation). It is thus possible that immunologically activated macrophages (by virus infection or BCG infection) may be virucidal and that this function may play a role in resistance to HSV-1 in genetically resistant mice. Studies are in progress to determine whether this is the case.

ACKNOWLEDGMENTS

This study was supported in part by Public Health Service grants CA-08748 and AI-14832 from the National Cancer Institute and the National Institute of Allergy and Infectious Diseases, respectively.

LITERATURE CITED

1. **Bahn, A. K.** 1972. Basic medical statistics, p. 144–156. Grune and Stratton, New York.
2. **Berlinger, N. T., C. Lopez, M. Lipkin, J. Vogel, and R. A. Good.** 1977. Defective recognitive immunity in family aggregates of colon carcinoma. J. Clin. Invest. **59**:761–769.
3. **Blanden, R. V.** 1968. Modification of macrophage function. RES J. Reticuloendothel. Soc. **5**:179–202.
4. **Cohn, Z. A., and B. Benson.** 1965. The differentiation of mononuclear phagocytes. Morphology, cytochemistry and biochemistry. J. Exp. Med. **121**:153–167.
5. **Conrad, R. E., L. C. Yang, and H. B. Herscowitz.** 1977. Mononuclear phagocytic cells in peritoneal exudate of rabbits: a comparison of inducing agents. RES J. Reticuloendothel. Soc. **21**:103–107.
6. **Daniels, C. A., E. S. Kleinerman, and R. Snyderman.** 1978. Abortive and productive infections of human mononuclear phagocytes by type 1 herpes simplex virus. Am. J. Pathol. **91**:119–136.
7. **Dannenberg, A. M., Jr., M. Ando, and K. Shima.** 1972. Macrophage accumulation, division, maturation and digestive, and microbicidal capacities in tuberculous lesions. III. The turnover of macrophages and its relation to their activation and anti-microbial immunity in primary BCG lesions and those of reinfection. J. Immunol. **109**:1109–1121.
8. **Edelson, P. J., and Z. A. Cohn.** Effects of concanavalin A on mouse peritoneal macrophages. I. Stimulation of endocytic activity of phagolysosome formation. J. Exp. Med. **140**:1364–1385.
9. **Edelson, P. J., and Z. A. Cohn.** 1976. 5'-Nucleotidase activity of mouse peritoneal macrophages. I. Synthesis and degradation in resident and inflammatory populations. J. Exp. Med. **144**:1581–1594.
10. **Edelson, P. J., R. Zwiebel, and Z. A. Cohn.** 1975. The pinocytic rate of activated macrophages. J. Exp. Med. **142**:1150–1164.
11. **Hirsch, M. S., B. Zisman, and A. C. Allison.** 1970. Macrophages and age-dependent resistance to herpes simplex virus in mice. J. Immunol. **104**:1160–1165.
12. **Johnson, R. T.** 1964. The pathogenesis of herpes virus encephalitis. II. A cellular basis for the development of resistance with age. J. Exp. Med. **120**:359–371.
13. **Lopez, C.** 1975. Genetics of natural resistance to herpesvirus infections in mice. Nature (London) **258**:152–153.
14. **Lopez, C., and R. J. O'Reilly.** 1977. Cell-mediated immune responses in patients with recurrent herpes simplex infection. I. Lymphocyte proliferation assay. J. Immunol. **118**:895–901.
15. **Mackaness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. **116**:381–405.
16. **Mackaness, G. B.** 1969. The influence of immunologically commited lymphoid cells on macrophage activity *in vivo*. J. Exp. Med. **129**:973–991.
17. **Michell, R. H., S. J. Pancake, J. Noseworthy, and M. L. Karnovsky.** 1969. Measurement of rates of phagocytosis. J. Cell. Biol. **40**:216–224.
18. **Nathan, C. F., R. Asofsky, and W. D. Terry.** 1977. Characterization of the non-phagocytic adherent cell from the peritoneal cavity of normal and BCG-treated mice. J. Immunol. **118**:1612–1620.
19. **Reif, A. E., and J. M. V. Allen.** 1964. The AKR thymic antigens and its distribution in leukemia and nervous tissue. J. Exp. Med. **120**:413–432.
20. **Starr, S. E., A. M. Visintine, O. M. Tomeh, and A. J. Nahmias.** 1976. Effects of immunostimulants on resistance of newborn mice to herpes simplex type 2 infection. Proc. Soc. Exp. Biol. Med. **152**:57–60.
21. **Stevens, J. G., and M. L. Cook.** 1971. Restriction of herpes simplex virus by macrophages. J. Exp. Med. **133**:19–36.
22. **Stubbs, M., A. V. Kuhner, E. A. Glass, J. R. David, and M. L. Karnovsky.** 1973. Metabolic and functional studies on activated mouse macrophages. J. Exp. Med. **137**:537–541.
23. **Zisman, B., M. S. Hirsch, and A. C. Allison.** 1970. Selective effects of anti-macrophage serum, silica and anti-lymphocyte serum on pathogenesis of herpes virus infection of young adult mice. J. Immunol. **104**: 1155–1159.