INFECTION AND IMMUNITY, June 1980, p. 1028-1032
0019-9567/80/06-1028/05$02.00/0

Vol. 28, No. 3

Marrow-Dependent Cells Depleted by ^{89}Sr Mediate Genetic Resistance to Herpes Simplex Virus Type 1 Infection in Mice

CARLOS LOPEZ[1]*, ROBERT RYSHKE,[1] AND MICHAEL BENNETT[2]

Laboratory of Herpes Virus Infections, Sloan-Kettering Institute for Cancer Research, New York, New York 10021,[1] and Department of Pathology, Boston University School of Medicine, Boston, Massachusetts 02118[2]

Adult mice resistant to infection with 10^6 plaque-forming units of a virulent strain of herpes simplex virus type 1 were treated with ^{89}Sr to abrogate marrow-dependent cell functions. Treated mice were found to be much more susceptible to the herpes simplex virus type 1 infection than untreated mice. The virus persisted in the visceral tissues of ^{89}Sr-treated mice for 3 or more days postinfection but not in those of untreated mice. The virus also spread to the spinal cords of treated but not untreated mice. A marrow-dependent cell appeared to mediate resistance to herpes simplex virus type 1 by controlling the infection early after inoculation and not allowing the infection to spread to the central nervous system.

The immunobiology and genetics of resistance to infection with herpes simplex virus type 1 (HSV-1) and resistance to transplants of allogeneic or incompatible bone marrow have many similar properties. These include the following: (i) strain distribution of resistant and susceptible mice (4, 5, 12); (ii) regulation of resistance by two independent dominant genes not linked to the major histocompatibility complex (4, 13); (iii) genetic resistance, a property of hemopoietic cells as determined by marrow cell transfer experiments (5, 13); (iv) maturation of resistance at 3 weeks of age (5, 8); and (v) genetic resistance impaired in each case by macrophage poisons (13, 15, 21). In addition, studies by Bennett (1) have shown that resistance to allogeneic marrow is a marrow-dependent trait as the treatment of mice with ^{89}Sr abrogates this resistance. ^{89}Sr treatment of mice reduces a marrow-dependent cell (M cell) function without impairing the capacity of the host to reject skin grafts or make antibody in response to antigenic challenge (2). We report here that the depletion of M cell function by the treatment of adult, resistance mice with ^{89}Sr also abrogates genetic resistance to HSV-1. The M cell appears to mediate resistance by controlling HSV-1 infection during the first 3 days postinfection and not allowing the virus to reach the central nervous system.

MATERIALS AND METHODS

Mice. (C57BL/6 × DBA/2)F_1 (BDF1) mice were obtained from The Jackson Laboratories, Bar Harbor, Maine. These mice were injected intravenously with 100 μCi of ^{89}Sr (Oak Ridge National Laboratory, Oak Ridge, Tenn.) on each of two separate occasions 4 weeks apart and were used in these experiments 1 to 2 months after the last injection. BDF1 mice of the same age (3 to 4 months) and sex served as controls.

Virus. HSV-1 strain 2931 (12) was used throughout these studies. This virus was propagated on Vero cell monolayers and quantitated on the same cells by the plaque assay (12). HSV-1 (2931) was inoculated intraperitoneally. Various tissues (brain, spinal cord, spleen, liver, and kidney) were harvested at various times post-inoculation (PI) for histopathological examination and for quantitation of the virus. Tissues were fixed in Bouin fixative and stained with hematoxylin and eosin. A portion of each tissue was used to make a 10 or 20% (wt/vol) suspension in Dulbecco-modified Eagle media. After grinding in a morter with pestle and sterile sand, the suspensions were clarified by centrifugation at 300 × *g* for 20 min and stored frozen (−70°C) until quantitated. Monolayers of Vero cells in small (35 × 10 mm) petri dishes were inoculated to determine the number of plaque-forming units (PFU) per gram of tissue. At least two and usually three or four mice were harvested for each datum point. The virus concentration was determined for each tissue from each mouse. When the tissues from more than one mouse in a group of two to four mice contained HSV-1, the logs of the concentrations were used to develop a mean virus concentration and standard deviation.

Immunofluorescence. Tissues, which were to be evaluated for HSV-1 antigen by the immunofluorescence technique, were snap frozen in isopentane and Dry Ice, embedded in 1% gelatin, and sectioned with a cryostat. Sections were flooded for 30 min at 37°C with antisera with a high neutralizing antibody titer (1:64) to HSV-1, after adsorption with either HSV-1-infected cells or -uninfected Vero cells. After thorough washing in two changes of cold phosphate-buffered saline, the sections were flood with fluorescein isothiocyanate-labeled anti-human gamma globulins (Hyland Laboratories, Inc., Costa Mesa, Calif.) for 30 min at 37°C. After thorough washing, the slides were dried, and the sections were covered with glycerol-phosphate-buffered saline and a cover slip. Sections were viewed with a Leitz orthoplan microscope.

RESULTS

^{89}Sr-treated and untreated BDF1 mice were inoculated with 10^6, 10^5, or 10^4 PFU of HSV-1 (2931) and observed for resistance over a 3-week period. As expected (12), the untreated BDF1 mice were resistant to as much as 10^6 PFU of this virulent strain of HSV-1. All ^{89}Sr-treated mice inoculated with as little as 10^3 PFU of HSV-1 (2931) succumbed to the infection. Moreover, the ^{89}Sr-treated mice died at 5 to 7 days PI and at the same time PI that the genetically most susceptible mice usually died. The ^{89}Sr-treated mice often died before signs of the infection were noted. Others, however, demonstrated the typical hind leg paralysis and "humpback" appearance before death (12, 20).

Three additional groups of ^{89}Sr-treated and untreated BDF1 mice were inoculated with HSV-1 (2931) for a histopathological study, for a study of the distribution of the virus in the tissues of these mice, and for an immunofluorescent study of virus antigen in spinal cord tissue.

Histopathological studies. Spleens, livers, brains, spinal cords, and kidneys of ^{89}Sr-treated and untreated BDF1 mice inoculated with 10^5 PFU of HSV-1 (2931) were harvested at 3 to 5 days PI. When compared with tissues from untreated mice either inoculated with HSV-1 or not, the tissues of the ^{89}Sr-treated, HSV-1-inoculated mice were indistinguishable. Specifically, cytological evidence of damage to liver, spinal cord, or brain tissues of ^{89}Sr-treated, HSV-1-infected mice was minimal or undetectable.

Pathogenesis studies. ^{89}Sr-treated and untreated mice were inoculated with either 10^5 or 10^3 PFU of HSV-1 (2931) and were sacrificed at 1, 3, or 5 days PI. Spleen, liver, kidney, spinal cord, and brain tissues were harvested for quantitation of the virus in each tissue (Table 1). In mice inoculated with 10^5 PFU, HSV-1 persisted on days 3 and 5 PI in the spleen and liver tissues only of the ^{89}Sr-treated mice, whereas the virus persisted in the kidney tissues of treated and untreated mice. Similarly, in mice inoculated with 10^3 PFU of HSV-1 (2931), the virus persisted in the spleen, liver, and kidney tissues on day 5 PI but did not persist in the tissues of untreated mice. In addition, HSV-1 was isolated from the spinal cord tissues of 4 out of 5 and from the brain tissues of 2 out of 5 ^{89}Sr-treated mice but not from spinal cord tissues of untreated mice (Table 1). One plaque, typical of HSV-1, was found in the brain tissue of one untreated, HSV-1-infected mouse. It was of interest to us that HSV-1 could be isolated from the spinal cord tissues of ^{89}Sr-treated, HSV-1-infected mice, whereas the same tissues demonstrated minimal histopathological evidence of infection. An additional experiment was carried out with the indirect immunofluorescence test to localize virus antigen in spinal cord tissues.

Immunofluorescence. The spinal cords were harvested from two ^{89}Sr-treated and two untreated BDF1 mice at 6 days PI with 10^6 PFU of HSV-1 (2931). Sections were stained with an antiserum with a high neutralizing antibody titer to HSV-1 after adsorption with either HSV-1-infected cells or uninfected Vero cells. Only the spinal cord tissues from the two ^{89}Sr-treated mice demonstrated fluorescense with the Vero cell-adsorbed antisera but not (or very little) with the HSV-1-adsorbed antisera (Fig. 1). Spinal cord tissues from untreated mice were uniformly negative. The immunofluorescence was found either as brightly fluorescing, large individual cells scattered throughout the tissue or as patches of fluorescing cells (Fig. 1). Since little histopathological evidence of infection had been seen earlier, sections demonstrating fluorescence were subsequently fixed for staining with hematoxylin and eosin. Although the quality of the preparation was compromised by the preparation procedures, there was clearly little evidence of the characteristic myelitis usually found in HSV-1-infected mice (20).

DISCUSSION

These data support the hypothesis that M cells function in an important way to prevent fatal HSV-1 infections of mice. Treatment of mice with the bone-seeking isotope, ^{89}Sr, chronically irradiates the bone marrow with high-energy beta particles and causes aplasia. The spleen takes over stem cell functions of the body and provides B cells, T cells, and accessory (macrophage) cells necessary for antibody responses and cell-mediated immune responses (2). However, mice treated with ^{89}Sr lose genetic resistance to bone marrow allografts (1, 2), genetic resistance to the leukemogenic and immunosuppressive effects of Friend erythroleukemia virus (2, 11), and resistance to early stages of infection with the facultative intracellular bacterium, *Listeria monocytogenes* (2). On the other hand, M cells do not function to resist the extracellular organism *Yersina pestis* (2). In addition, ^{89}Sr treatment decreases natural killer cell function (7), and M cells appear to restrict the function or numbers of suppressor cells or both (11, 17). It thus appears that the M cell system plays a central role in the defense of the host against various microorganisms.

Our study of the pathogenesis of HSV-1 (2931) infection of ^{89}Sr-treated mice indicates that the virus persists in spleen and liver tissues of ^{89}Sr-treated but not untreated mice. In addition, the

TABLE 1. *Isolation of HSV-1 from tissues of ^{89}Sr-treated and untreated mice inoculated with 10^3 or 10^5 PFU of HSV-1 (2931)*[a]

Tissue	HSV-1[b] isolated at days PI		
	1	3	5
10^5 PFU inoculum			
Spleen			
Untreated	2/3 (3.75 ± 1.6)	0/3	0/2
^{89}Sr-treated	3/3 (2.96 ± 2.1)	1/3 (2.3)	2/2 (2.37 ± 0.76)
Liver			
Untreated	3/3 (3.55 ± 1.11)	0/3	0/2
^{89}Sr-treated	3/3 (2.88 ± 2.08)	1/3 (2.48)	2/2 (2.2 ± 1.01)
Kidney			
Untreated	ND[c]	1/3 (3.48)	1/2 (2.85)
^{89}Sr-treated	ND	2/3 (2.02 ± 0.03)	2/2 (3.12 ± 0.49)
10^3 PFU inoculum			
Spleen			
Untreated	ND	ND	0/2
^{89}Sr-treated	ND	4/4 (2.92 ± 0.53)	4/5 (2.7 ± 0.53)
Liver			
Untreated	ND	ND	0/2
^{89}Sr-treated	ND	3/4 (2.0 ± 0.61)	3/5 (2.8 ± 1.2)
Kidney			
Untreated	ND	ND	0/2
^{89}Sr-treated	ND	3/4 (3.14 ± 0.24)	4/5 (≫4.0)
Spinal cord			
Untreated	ND	ND	0/2
^{89}Sr-treated	ND	0/4	4/5 (2.48 ± 1.3)
Brain			
Untreated	ND	ND	1/2 (0.84)[d]
^{89}Sr-treated	ND	0/4	2/5 (1.73 ± 0.37)

[a] Tissues were harvested at 1, 3, or 5 days PI.

[b] Number of tissues positive out of total tested. Numbers in parentheses represent the mean log of virus concentration per gram of tissue ± the standard deviation.

[c] ND, Not done.

[d] 1 PFU found.

virus was detected in the spinal cords of ^{89}Sr-treated mice (6 out of 7; 4 out of 5 by virus isolation and 2 out of 2 by immunofluorescence) but not in the spinal cords of untreated mice (0 out of 4; 2 by virus isolation and 2 by immunofluorescence). Lastly, HSV-1 was isolated from 2 out of 5 brain tissues of ^{89}Sr-treated mice, whereas only 1 PFU was found in brain tissues of two untreated mice. These studies thus indicate that the result of the abrogation of M cell function in HSV-1-infected mice is that the viral infection persists in the visceral tissues and travels to the spinal cord. The latter is probably accomplished via the nervous system rather than hematogenously as much less virus was found in brain tissue. The M cell system is therefore apparently responsible for clearing the viral infection during the early stages of infection and not allowing it to spread into or through the nervous system. In this regard, it is interesting to note that Minato et al. (18) have recently found that natural killer cells, also thought to be related to the M cells (10), play an important role in resistance to persistent virus infections. Further support for this conclusion is our recent finding of low natural killer cell function in patients with severe, recurrent herpesvirus infections (3).

There are similarities between the M cell and the macrophage which suggest that they may be the same or closely related cells. For example, the functions of both of these cells are diminished after in vivo treatment with silica particles (15) and carrageenan (6), both are relatively

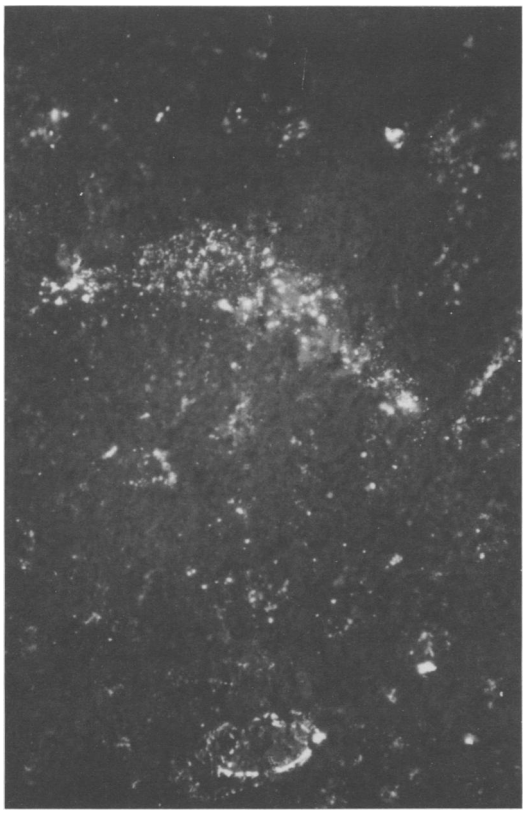

FIG. 1. *HSV-1-specific fluorescence demonstrated in frozen section of spinal cord tissue of ^{89}Sr-treated, HSV-1-infected mouse. Spinal cord tissue was harvested from ^{89}Sr-treated mouse at 6 days PI with 10^6 PFU of HSV-1. The tissue was snap frozen and sectioned with a cryostat, and the sections were fixed in cold acetone. Specificity was determined by absorption of the activity with HSV-1-infected cells but not with uninfected Vero cells.*

resistant to gamma irradiation (5), and both have been associated with resistance to *L. monocytogenes* (2, 16) and HSV-1 (8, 9, 21) infections. There are, on the other hand, arguments against their being the same cell as follows, (i) ^{89}Sr treatment of mice abrogates resistance to *L. monocytogenes* and HSV-1 without affecting the humoral or cell-mediated immune capacities (2) and, therefore, without severely diminishing the accessory cell function of the macrophage; (ii) ^{89}Sr treatment of mice abrogates resistance but does not result in necrotizing hepatitis after infection with HSV-1, whereas even small doses of silica (3 mg), a macrophage poison, result in liver lesions after infection with HSV-1 (19); and (iii) although the capacity of macrophages to restrict HSV-1 replication has been shown to play a major role in the defense against infection in the adult versus the newborn mouse (9), comparable studies of genetically resistant versus susceptible mice indicate that this macrophage function does not segregate with resistance and cannot account for such defense (14). These studies, in summary, suggest that, although the M cell and the macrophage are probably not the same cell, they may be related or interact with each other in natural resistance systems.

The relative lack of the classical histopathological finding of myelitis in the spinal cord tissues of ^{89}Sr-treated, HSV-1-infected mice was unexpected. It is possible that such a response might require more time to develop than was allowed in this study. It is also possible that abrogation of M cell function might have diminished the inflammatory response often found with the viral infection.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grants CA-08748, AI-14832, CA-15369, and CA-15848 from the National Institutes of Health. C.L. is the recipient of Faculty Research Award ACS FRA 193. M.B. is the recipient of Research Career Development Award NIH CA-70879.

LITERATURE CITED

1. **Bennett, M.** 1973. Prevention of marrow allograft rejection with radioactive strontium: evidence for marrow-dependent effector cells. J. Immunol. **110**:510–516.
2. **Bennett, M., E. E. Baker, J. W. Eastcott, V. Kumar, and D. Yonkosky.** 1976. Selective eliminations of marrow precursors with the bone-seeking isotope ^{89}Sr: implications for hemopoiesis, lymphopoiesis, viral leukemogenesis and infection. RES J. Reticuloendothel. Soc. **20**:71.
3. **Ching, C., and C. Lopez.** 1979. Natural killing of herpes simplex virus type 1-infected target cells: normal human responses and influence of antiviral antibody. Infect. Immun. **26**:49–56.
4. **Cudkowicz, G.** 1975. Genetic control of resistance to allogeneic and xenogeneic bone-marrow grafts in mice. Transplant. Proc. **7**:155–159.
5. **Cudkowicz, G., and M. Bennett.** 1971. Peculiar immunobiology of bone marrow allografts. I. Graft rejection by irradiated responder mice. J. Exp. Med. **134**:83–102.
6. **Cudkowicz, G., and Y. P. Yung.** 1977. Abrogation of resistance to foreign bone marrow grafts by carrageenans. I. Studies with the antimacrophage agent Seakem carrageenan. J. Immunol. **119**:483–487.
7. **Haller, O., and H. Wigzell.** 1977. Suppression of natural killer cell activity with radioactive strontium: effector cells are marrow dependent. J. Immunol. **118**:1503–1506.
8. **Hirsch, M. S., B. Zisman, and A. C. Allison.** 1970. Macrophage and age dependent resistance to herpes simplex virus in mice. J. Immunol. **104**:1160–1165.
9. **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**:359–374.
10. **Kiessling, R., P. S. Hochman, O. Haller, G. Shearer, H. Wigzell, and G. Cudkowicz.** 1977. Evidence for a similar or common mechanism for natural killer cell activity and resistance to hemopoietic grafts. Eur. J. Immunol. **7**:655–662.
11. **Kumar, V., M. Bennett, and R. J. Eckner.** 1974. Mech-

anisms of genetic resistance to Friend virus leukemia in mice. I. Role of ^{89}Sr-sensitive effector cells responsible for rejection of bone marrow allografts. J. Exp. Med. **139**:1093–1109.

12. **Lopez, C.** 1975. Genetics of natural resistance to herpesvirus infections in mice. Nature (London) **258**:152–153.
13. **Lopez, C.** 1978. Immunological nature of genetic resistance of mice to herpes simplex virus type-1 infection, p. 775–778. *In* G. De The, W. Henle, and F. Rapp (ed.), Oncogenesis and herpesviruses III. International Agency for Research, Lyon, France.
14. **Lopez, C., and G. Dudas.** 1979. Replication of herpes simplex virus type 1 in macrophages from resistant and susceptible mice. Infect. Immun. **23**:432–437.
15. **Lotzova, E., and G. Cudkowicz.** 1974. Abrogation of resistance to bone marrow grafts by silica particles. Prevention of the silica effect by the macrophage stabilizer poly-2-vinylpyridine *N*-oxide. J. Immunol. **113**: 798–803.
16. **Mackaness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. **116**:381–406.
17. **Merluzzi, V. J., E. M. Levy, V. Kumar, M. Bennett, and S. R. Cooperband.** 1978. *In vitro* activation of suppressor cells from spleens of mice treated with radioactive strontium. J. Immunol. **121**:505–512.
18. **Minato, N., B. R. Bloom, C. Jones, J. Holland, and L. M. Reid.** 1979. Mechanisms of rejection of virus persistently infected tumor cells by athymic nude mice. J. Exp. Med. **149**:1117–1133.
19. **Mogensen, S. C., and H. K. Andersen.** 1977. Effect of silica on the pathogenic distinction between herpes simplex virus type 1 and 2 hepatitis in mice. Infect. Immun. **17**:274–277.
20. **Walz, M. A., R. W. Price, K. Hayashi, B. J. Kàtz, and A. L. Notkins.** 1977. Effect of immunization on acute and latent infections of vaginouterine tissue with herpes simplex virus types 1 and 2. J. Infect. Dis. **135**:744–752.
21. **Zisman, B., M. S. Hirsch, and A. C. Allison.** 1970. Selective effects of anti-macrophage serum, silica, and anti-lymphocyte serum on the pathogenesis of herpesvirus infection in young adult mice. J. Immunol. **104**: 1155–1159.