PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 29-32 (1978)

Resistance of C3H/HeJ Mice to Lethal Challenge with Herpes Simplex Virus (39983)

HOLGER KIRCHNER, HANS MARTIN HIRT, DAVID L. ROSENSTREICH, AND STEPHAN E. MERGENHAGEN[1]

Institute of Virology, German Cancer Research Center, Heidelberg, FRG, and Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014

Introduction. Herpes simplex virus (HSV) replicates *in vitro* in spleen cells of certain strains of mice after prestimulation with mitogenic doses of endotoxic lipopolysaccharide (LPS) (1). However, no viral replication occurs in unstimulated cultures or in cultures prestimulated with T-cell mitogens, suggesting that B lymphocytes or macrophages activated by LPS are the target cells for HSV replication in mouse spleen cell cultures.

In order to provide more definitive data on the role of lymphoreticular cells in HSV replication *in vitro* we recently employed endotoxin-unresponsive mice (C3H/HeJ) in our experiments. This mouse strain is resistant to a number of toxic and immunological effects of LPS (2-5). Furthermore, both the B lymphocytes and macrophages of this strain are unresponsive to LPS (6, 7). As anticipated, spleen cells from C3H/HeJ mice pretreated with LPS failed to proliferate and did not support virus replication *in vitro* in the same manner as did spleen cells from the closely related C3HeB/FeJ mouse which is susceptible to the biological effects of LPS. We therefore compared the susceptibility of these two strains of mice to *in vivo* infection with HSV.

Materials and methods. Virus. HSV-1 (WAL) strain was adapted to mouse brain and used for *in vivo* experimentation (8). A pool of infected brains was prepared, homogenized, diluted 1/10 in saline, and frozen in aliquots at −70°. This pool, containing 5×10^6 plaque-forming units (PFU)/ml, was used for all experiments. Before use, cellular material was removed by centrifugation. For *in vitro* experiments, a tissue culture-derived preparation of HSV (WAL) grown in VERO cells and containing 5×10^8 PFU/ml was used. Viral titrations were performed in mouse embryo fibroblasts (MEF) which were derived from one batch of frozen cells and used between the third and ninth passage. The detail of the viral plaque assay have been described (1).

Mice. Male C3HeB/FeJ and C3H/HeJ mice, 8-16 weeks old, obtained from Jackson Laboratories (Bar Harbour, Maine) were injected with 10-fold serial dilutions of HSV in 0.1-ml (intraperitoneally, ip) or in 0.03-ml (intracerebrally, ic) aliquots. Deaths were recorded daily until 18 days after infection and the LD_{50} was calculated as described (8).

Spleen cell cultures. Single spleen cell suspensions were prepared using sterile technique and stimulated by mitogens in a standard lymphocyte stimulation assay (1). The following mitogens were used: PHA (purified phytohemagglutinin; Wellcome Laboratories, Bechenham, England, 2.5 μg/ml), Con A (concanavalin A; Calbiochem, San Diego, Calif., 2.5 μg/ml), and LPS (lipopolysaccharide W *Salmonella typhimurium* 3125-25; Difco, Detroit, Mich., 10 μg/ml). A crude preparation of *Acholeplasma laidlawaii* (AL) was prepared and used as a mitogen (submitted for publication).

For demonstration of viral replication in mitogen-stimulated spleen cell cultures a previously described technique was followed (1). After prestimulation with optimally stimulatory doses of mitogens for 2 days, cultures were infected with HSV-1 at a multiplicity of infection of 10. After 24 hr, the cultures were terminated by rapid freezing at −70°. Viral titers were determined by plaque assay in MEF (see above).

Peritoneal exudate cells. Mice were in-

[1] This work was initiated when S.E.M. was a Visiting Professor at the University of Heidelberg. Send reprint requests to Dr. Stephan E. Mergenhagen, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20014.

0037-9727/78/1571-0029$01.00/0

jected ip wth 10^5 PFU of HSV and sacrificed after 24 hr. Five milliliters of saline was injected ip and the cells of the peritoneal cavity (PEC) were recovered by standard techniques. The PEC were centrifuged, resuspended in medium, and directly plated on confluent monolayers of MEF for evaluation by the viral plaque assay.

Results. In vitro mitogenicity of LPS for spleen cells of C3H/HeJ and C3HeB/FeJ mice and replication of HSV in these cultures. As previously described by several laboratories (3, 5) spleen cells of C3H/HeJ mice did not respond to the mitogenic effect of LPS in culture, in contrast to spleen cells from C3HeB/FeJ mice (Table I). However, they responded well to AL, another B cell mitogen (unpublished observation) and to the T-cell mitogens PHA and Con A. The ability of C3H/HeJ mice to respond to B-cell mitogens other than LPS is well known (3).

We have previously reported that replication of HSV in mouse spleen cultures required lymphoid cell activation with a B-cell mitogen such as LPS (1). We therefore investigated whether HSV would replicate *in vitro* in C3H/HeJ spleen cells. There was no demonstrable HSV replication in LPS-treated C3H/HeJ cells ($<10^1$ PFU/ml), while HSV replicated very well in LPS-treated C3HeB/FeJ spleen cells (4.2×10^3 PFU/ml) (Table II). However, in spleen cell cultures of C3H/HeJ mice stimulated with the B-cell mitogen, AL, virus replication did occur (5.3×10^3 PFU/ml), thus demonstrating that C3H/HeJ spleen cells were capable of replicating HSV *in vitro* when appropriately stimulated.

TABLE I. EFFECT OF DIFFERENT MITOGENS ON SPLEEN CELL CULTURES OF C3H/HeJ AND C3HeB/FeJ MICE

	Lymphocyte proliferative response (cpm [^{3}H]TdR incorporated/6×10^5 cells)	
Mitogen	C3H/HeJ	C3HeB/FeJ
None added	1,733	1,991
PHA	63,774	74,954
Con A	101,301	111,419
LPS	1,946	45,745
AL	25,613	23,420

TABLE II. *In vitro* REPLICATION OF HSV IN SPLEEN CELL CULTURES OF C3HeB/FeJ AND C3H/HeJ MICE.

	HSV replication[a] (PFU/3×10^6 cells)	
Mitogen	C3H/HeJ	C3HeB/FeJ
None added	$<10^1$	$<10^1$
PHA	$<10^1$	$<10^1$
LPS	$<10^1$	4.2×10^3
AL	5.3×10^3	1.2×10^3

[a] Cultures were preincubated with or without mitogen for 48 hr, infected with HSV, and incubated for another 24 hr. Titers were determined in MEF in a viral plaque assay.

Lethality of C3H/HeJ and C3HeB/FeJ mice after ip and ic infection with HSV. We next determined the intrinsic susceptibility of these mice to ip challenge with HSV in the absence of any exogenously administered LPS. Quite surprisingly, we found that approximately 100 times more HSV was required to kill C3H/HeJ mice after ip inoculation than to kill C3HeB/FeJ mice (Table III).

It is thought that after ip inoculation, HSV first replicates in the peritoneum, then travels via the blood to the brain where it produces a fatal encephalitis (9). In order to determine if C3H/HeJ mice were resistant to HSV because of a failure of the virus to replicate in the cells of the target organ (brain), HSV was inoculated directly ic into both mouse strains. After ic inoculation, there was no significant difference in susceptibility between the two strains, although the LD_{50} was lower in both (Table III).

These findings suggested that the resistance of C3H/HeJ mice was due to a failure of HSV to replicate intraperitoneally. In order to determine if this was the case, 5×10^5 PFU of HSV were inoculated ip into both strains and virus titers were quantitated in their peritoneal cells 24 hr later. Viral recovery was more consistent and considerably higher from PEC of C3HeB/FeJ mice than from PEC of C3H/HeJ mice (Table IV). In four of six C3H/HeJ mice, no HSV was recovered from their PEC, and the remaining two mice yielded 5 and 17 PFU of HSV/ml, respectively. In contrast, six of six C3HeB/FeJ PEC contained HSV ranging in titer from 30 to 200 PFU/

TABLE III. LETHALITY OF C3H/HeJ AND C3HeB/FeJ MICE AFTER IP AND IC INFECTION WITH HSV.

Expt. No.	Type of infection	LD_{50} of HSV (PFU/mouse) C3H/HeJ	C3HeB/FeJ	P^a
I	ip	7.95×10^5	6.30×10^3	<0.05
II	ip	1.72×10^5	2.35×10^3	<0.05
III	ip	1.35×10^5	2.45×10^3	<0.05
IV	ic	1.78×10^2	4.27×10^2	Not significant
V	ic	4.27×10^1	3.20×10^2	Not significant

[a] Statistical evaluation performed using Student's *t* test.

TABLE IV. TITERS OF HSV IN PEC OF C3H/HeJ AND C3HeB/FeJ MICE 24 hr AFTER INFECTION OF 5 $\times 10^5$ PFU OF HSV.

Expt. No.	Mouse No.	Recovery of HSV from peritoneal cells (PFU/ml)[a] C3H/HeJ	C3HeB/FeJ
I	1	0.5×10^1	8×10^1
	2	0	1×10^2
	3	0	7×10^1
II	1	1.7×10^1	2×10^2
	2	0	9×10^1
	3	0	3×10^1

[a] PEC were recovered from the peritoneal cavity, thoroughly washed, suspended in medium, and tested on confluent monolayers of MEF in a viral plaque assay.

ml. Thus, these findings suggest that the resistance of C3H/HeJ mice to HSV is related to a failure of the virus to replicate intraperitoneally in this strain.

Discussion. We have recently described a system of *in vitro* replication of HSV in mouse spleen cell cultures prestimulated by LPS (1). Unstimulated spleen cell cultures and cultures stimulated by the T-cell mitogens PHA and Con A did not support viral growth. Since spleen cells of C3H/HeJ mice have been shown to be unreactive to the effects of LPS (3, 5), it is not surprising that replication of HSV could not be demonstrated in these cultures. This observation led us to compare the lethality of HSV in both strains of mice. In this report, we have shown that the mortality of C3HeB/FeJ mice after ip infection with HSV was significantly higher than that of C3H/HeJ mice. This difference appears to be noteworthy since, to our knowledge, it represents the first demonstration of a difference in susceptibility to a viral infection in these closely related strains of mice that were thought to differ only in their endotoxin responsiveness.

Host susceptibility to viral infections is obviously determined by a complex series of factors (10). In a recent study, Lopez suggested that resistance to HSV-1 in mice was determined by at least three genes (11). The C3H/HeJ mouse strain has been extensively studied since it is unresponsive to all known effects of endotoxin due to a mutation in a single autosomal gene that occurred some time between 1960 and 1965 (12, 13). Furthermore, this defect is highly specific, since it seems to be limited to the lipid A moiety of LPS (14). The C3H/HeJ and C3HeB/FeJ strains are closely related, and the only identified difference between them is their LPS responsiveness. Therefore, the fact that the C3H/HeJ strain is resistant to lethal infection with HSV-1 is extremely interesting since it raises the possibility that the gene that controls LPS sensitivity is also one of the genes responsible for susceptibility to HSV-1 infection.

Our evidence suggests that the resistance of C3H/HeJ mice to HSV is due to events taking place in the peritoneal cavity since HSV grows poorly in peritoneal cells of C3H/HeJ mice (Table IV), and since they appear to grow well intracerebrally in this strain (Table III). Furthermore, our evidence also suggests that resistance is due to some defect in the activation of cells, since spleen cells of this strain will replicate HSV normally *in vitro* when appropriately stimulated (Table II). Finally, that the connection between LPS and HSV sensitivity is significant and not merely coincidental is suggested by the findings that LPS is required for the replication of HSV *in vitro*, and that a mouse strain whose only known defective response is to LPS is also resistant to HSV.

While replication of HSV-1 within peritoneal cells appears to be one of the factors necessary for the induction of lethality, it is obviously not the only factor involved. HSV-1 replicates normally in (C57/BL/6 × DBA/2J) F1 mice even though these mice are resistant to its lethal effects (1). These findings are best explained by the hypothesis that more than one gene controls susceptibility to HSV-1 induced lethality (11).

It is not yet known whether HSV-1 replicates within the B lymphocytes or the macrophages in the peritoneal cavity. Since the responses of both cell types to LPS are abnormal in C3H/HeJ mice (7), either one or both cells could be the target. However, our previous findings suggest that the activated B cell is more important than the macrophage in this regard since HSV-1 replicates well in macrophage-depleted B lymphocytes but replicates only poorly in isolated macrophage populations (1).

It is premature to speculate on the relationship between the sensitivity of the mouse to HSV and LPS. First it must be demonstrated if this connection is real. A formal genetic analysis to ascertain whether the LPS gene controls HSV sensitivity is currently underway. However, if this does turn out to be the case, it would suggest that either LPS or some LPS-like molecule plays an important but heretofore unrecognized role in the homeostasis of the lymphoid system, or that the LPS gene regulates some important, non-LPS-related functions in the host. The implications of either possibility are potentially great.

Summary. In vitro replication of herpes simplex virus (HSV) in murine spleen cells requires simultaneous cell stimulation with a B-cell mitogen such as lipopolysaccharide (LPS). As expected, spleen cells of LPS-unresponsive C3H/HeJ mice did not support HSV replication in LPS-pretreated cultures, while spleen cells from closely related but LPS-responsive C3HeB/FeJ did. More importantly, the C3H/HeJ strain was found to be intrinsically resistant to HSV infection *in vivo*. After intraperitoneal (ip) inoculation, HSV was 50–120 times more virulent for C3HeB/FeJ mice than for the C3H/HeJ strain. This resistance appeared to be due to a failure of HSV to replicate in C3H/HeJ peritoneal cells, since after ip infection with HSV, recovery of virus was higher and more consistent from peritoneal exudate cells of C3HeB/FeJ mice than from C3H/HeJ mice. In addition, no difference in lethality was observed between these two strains after a direct intracerebral inoculation of HSV. This observation that LPS-unresponsive mice are intrinsically resistant to lethal HSV infection, coupled with the LPS requirement for HSV replication *in vitro*, suggests an important but as yet unexplained link between host sensitivity to HSV and to LPS.

1. Kirchner, H., Hirt, H. M., Kleinicke, C., and Munk, K., J. Immunol. **117,** 1753 (1976).
2. Rosenstreich, D. L., and Glode L. M., J. Immunol. **115,** 777 (1975).
3. Rosenstreich, D. L., and Mergenhagen, S. E., *in* "Microbiology 1975," p. 320. American Society of Microbiology (1975).
4. Sultzer, B. M., Infect. Immun. **5,** 107 (1972).
5. Watson, J., and Riblet, R. J. Immunol. **114,** 1462 (1975).
6. Glode, L. M., Scher, I., Osborne, B., and Rosenstreich, D. L., J. Immunol. **116,** 454 (1976).
7. Glode, L. M., Jacques, A., Mergenhagen, S. E., and Rosenstreich, D. L., J. Immunol., **119,** 162 (1977).
8. Kirchner, H., Hirt, H. M., and Munk, K., Infect. Immun. (in press) (1977).
9. Johnson, R. T., J. Exp. Med. **120,** 359 (1964).
10. Shif, I., and Bang, F. B., J. Exp. Med. **131,** 843 (1970).
11. Lopez, C., Nature (London) **258,** 152 (1975).
12. Watson, J., and Riblet, R., J. Exp. Med. **140,** 1147 (1974).
13. Glode, L. M., and Rosenstreich, D. L., J. Immunol. **117,** 2061 (1976).
14. Morrison, D. C., Betz, S. J., and Jacobs, D. M., J. Exp. Med. **144,** 840 (1976).

Received June 27, 1977. P.S.E.B.M. 1978, Vol. 157.