Zosteriform Spread of Herpes Simplex Virus as a Model of Recrudescence and Its Use to Investigate the Role of Immune Cells in Prevention of Recurrent Disease

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During the development of a zosteriform rash, which occurs after flank inoculation of BALB/c mice with herpes simplex virus, clinically normal skin becomes infected via nerve endings. This is analogous to the final step in the development of a recrudescent lesion, which may occur after reactivation of latent virus. Therefore, the zosteriform reaction has potential as a model with which to study the modification of such a recrudescent infection by immune processes. Using an adoptive transfer system, we confirmed that immune lymph node cells are potent in accelerating the clearance of virus from the primary site of replication (the inoculation site). This effect was T cell dependent. However, if injection of the same cell population was delayed until ganglionic infection was established, the appearance of the zosteriform rash was not prevented, and the virus titer recovered from the lower flank was not reduced. Immunoperoxidase studies showed that virus is at first highly localized to the epidermis after it emerges from nerves. As determined by conventional histology, little cellular infiltration was seen until clinical lesions were apparent. These observations indicate that recrudescent lesions appear in the presence of cell populations normally associated with rapid virus clearance; cellular immune mechanisms may be rendered ineffective owing to the lack of recruitment to the site of recrudescence until tissue breakdown instigates an inflammatory response.

In 1923 Teague and Goodpasture (26) observed that rabbits injected in the midflank with herpes simplex virus (HSV) developed a bandlike ipsilateral lesion several days later, akin to the eruption of shingles caused by varicella-zoster virus in humans. These authors concluded that virus travelled from the inoculation site into the sensory nervous system and then returned to the skin of the whole neurodermatome, resulting in the zosteriform rash.

This phenomenon has been reproduced in mice (2, 12, 25). Although not previously appreciated, this indicates that viral growth at any peripheral site may have a zosteriform component superimposed on initial local replication. After flank infection, part of this component is clearly distinguished as being remote from the inoculation site. However, at other sites (e.g., the ear pinna) a zosteriform component would not be clearly recognized as distinct, for anatomical reasons.

Immunity to HSV infection in experimental animals has been extensively investigated (18). In particular, the mouse ear model (6) has been used to probe in detail the effects of cellular immune mechanisms on the course of the primary infection (8, 13, 15–17). In these studies the assumption has been that the immune mechanisms were mediating their effect by rapidly destroying infected epidermal or other peripheral cells, thereby inhibiting cell-to-cell spread; a modification of the zosteriform component of growth has not been previously considered.

Replication of virus in skin infected via nerve endings can be investigated separately from replication at the inoculation site in mice infected in the flank. The importance of this point is that this is the route by which skin is infected after virus has reactivated in the nervous system. Although reactivation precedes true recrudescence, the development of a lesion requires infection and destruction of epidermal cells. Therefore, in mice infected in the flank we have available a

potential model in which immune modulation of the development of a recrudescent lesion can be studied.

We report here our virological findings with mice infected in the flank and demonstrate the importance of such a system in the study of immune processes active against primary and perhaps recrudescent HSV infection.

MATERIALS AND METHODS

Mice. Female inbred BALB/c mice raised in the Department of Pathology animal house were used throughout. They were infected at 6 to 8 weeks of age.

Virus. A well-characterized clone of a recent oral isolate of HSV type 1, strain SC16 (5), was grown in BHK-21 cells and stored in portions at $-70°C$ until it was required.

Inoculation of mice. Each left flank was clipped and depilated with Nair (Carter-Wallace Ltd., Folkestone, United Kingdom), and the mice were then lightly anesthetised with ether. Stock virus was diluted to 5×10^6 PFU/ml, and a 10-μl drop containing 5×10^4 PFU was placed on the skin of the posterior flank, just lateral to the spine, over the spleen. A total of 20 scarifications were made through the drop with a 27-gauge hypodermic needle, giving a total scarified area of approximately 4 mm^2.

Each donor for draining lymph node cells received 10^5 PFU of virus in 20 μl of Glasgow modified Eagle medium (GMEM) subcutaneously into the pinna as previously described (6).

Removal of tissue and infectious virus assay. A 0.5-cm-square piece of tissue encompassing the inoculation site was removed and placed into 1 ml of HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid)-buffered GMEM. The skin sample from the lower flank consisted of a 0.5-cm-wide strip of skin extending between the excised inoculation site and the anterior midline. To avoid contamination of this specimen with inoculated skin, a 0.5-cm section adjacent to
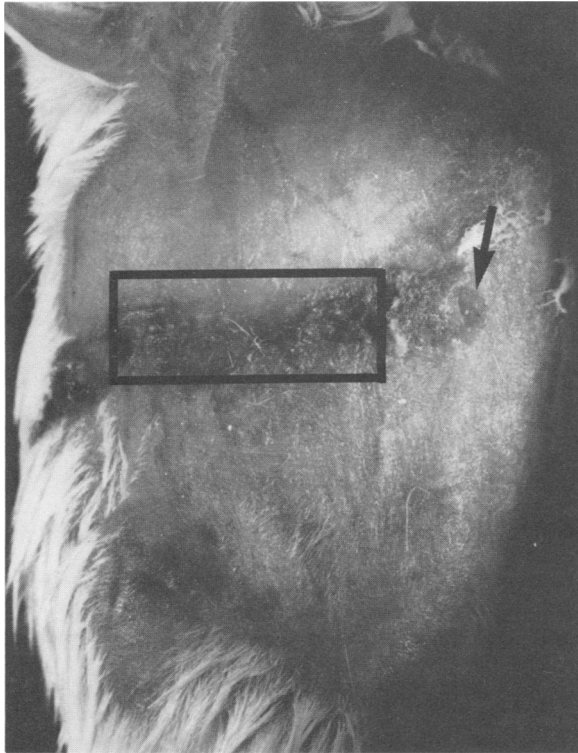
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FIG. 1. Left flank of a mouse 5 days after infection at one point (arrow). The zosteriform rash extends to the anterior midline. Outlined is the extent of the sample taken for virus assay. Note the gap left between this and the inoculation site.

the inoculation site was discarded. The instruments were washed in methylated spirits between each manipulation.

Thoracic dorsal root ganglia supplying the dermatome of inoculation were removed with the aid of an operating microscope. Specimens were stored in 1 ml of HEPES-buffered GMEM and frozen in −70°C until they were required.

To assay for the presence of infectious virus, tissue samples were homogenized in 1 ml of GMEM containing 10% calf serum and 10% tryptose phosphate broth; 10-fold dilutions were then tested for plaque production in BHK-21 cells (22).

Lymph node cell suspensions. Donor mice were killed 7 days after ear infection, and the nodes draining the infected pinnae were removed. A suspension containing 10^8 cells per ml in HEPES-buffered GMEM was prepared as previously described (13). Each recipient mouse received an intravenous dose of 200 μl of this preparation containing 2×10^7 cells.

T-cell depletion of lymph node cells. A total of 10^8 cells were pelleted and suspended in a dilution of a rat monoclonal anti-thy 1 antibody (designated YTS 154; a donation from H. Waldmann, Department of Pathology, University of Cambridge). The cells were incubated for 1 h on ice. Control cells were incubated with inactivated normal serum. The cells were again pelleted, suspended in rabbit serum, which was used as a source of complement, and incubated for 30 min at room temperature. Killing was determined by trypan blue staining; 36% of the antibody-treated cells were killed, compared with 6% nonspecific killing by complement alone. After a wash in phosphate-buffered saline, the surviving cells in each batch were donated to groups of five mice.

Immunoperoxidase test. The reagents used in the immunoperoxidase test were polyclonal sera kindly donated by P.G.H. Gell of our laboratory. The primary antiserum was rabbit anti-HSV, and the secondary antiserum was an anti-allotypic rabbit anti-rabbit immunoglobulin G. Peroxidase-rabbit anti-peroxidase conjugate was purchased from DAKO Immunoglobulins a/s. Calf serum was obtained from Sera Lab Ltd., Sussex, England.

Histological samples for testing were fixed in Bouin solution, processed routinely, and mounted in paraffin blocks. Sections (thickness, 5 μm) were cleared in xylene and rehydrated in graded alcohol solutions. Endogenous peroxidase was blocked with 0.1% hydrogen peroxide for 30 min at room temperature; the tissue was then washed and blocked with 20% calf serum for an additional 30 min.

The concentrations of the primary and secondary antisera and peroxidase-rabbit anti-peroxidase conjugate were initially optimized at room temperature for incubation periods of 1 h, 10 min, and 10 min, respectively. All subsequent staining was carried out by using these incubation times, followed by 3 min of immersion in a diaminobenzidine solution (50 mg/100 ml).

Sensory denervation of the left flank. Access to the sensory nerves of the flank was via a posterior midline incision between the first thoracic and first lumbar vertebrae. Anesthesia and analgesia were provided by a mixture of diazepam and fentanyl given intraperitoneally. Five nerves, which supplied the inoculation site and most of the adjacent flank, were sectioned in each mouse. Postoperatively, the efficacy of the operation was checked by demonstrating a lack of response to pinprick sensation. Control animals received a similar operation without nerve transection.

RESULTS

Isolation of virus from the skin of the flank. A total of 15 mice were infected in the flank, and groups of 3 were killed daily on days 1 to 5 postinfection (p.i.). A zosteriform lesion (day 5) is illustrated in Fig. 1; this lesion is exactly analagous
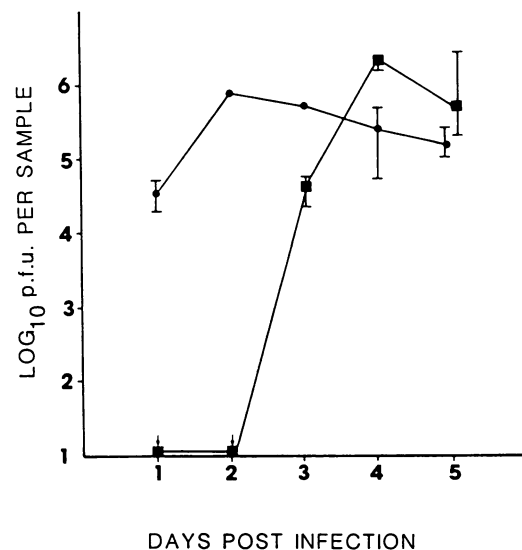

DAYS POST INFECTION

FIG. 2. Infectious virus titers recovered from the inoculation site (●) and lower flank (■) on varying days postinfection. Mice were given 5×10^4 PFU of HSV on day 0. Each point represents the arithmetic mean of values obtained from a group of three mice. Bars indicate ranges of values, where variation occurred.
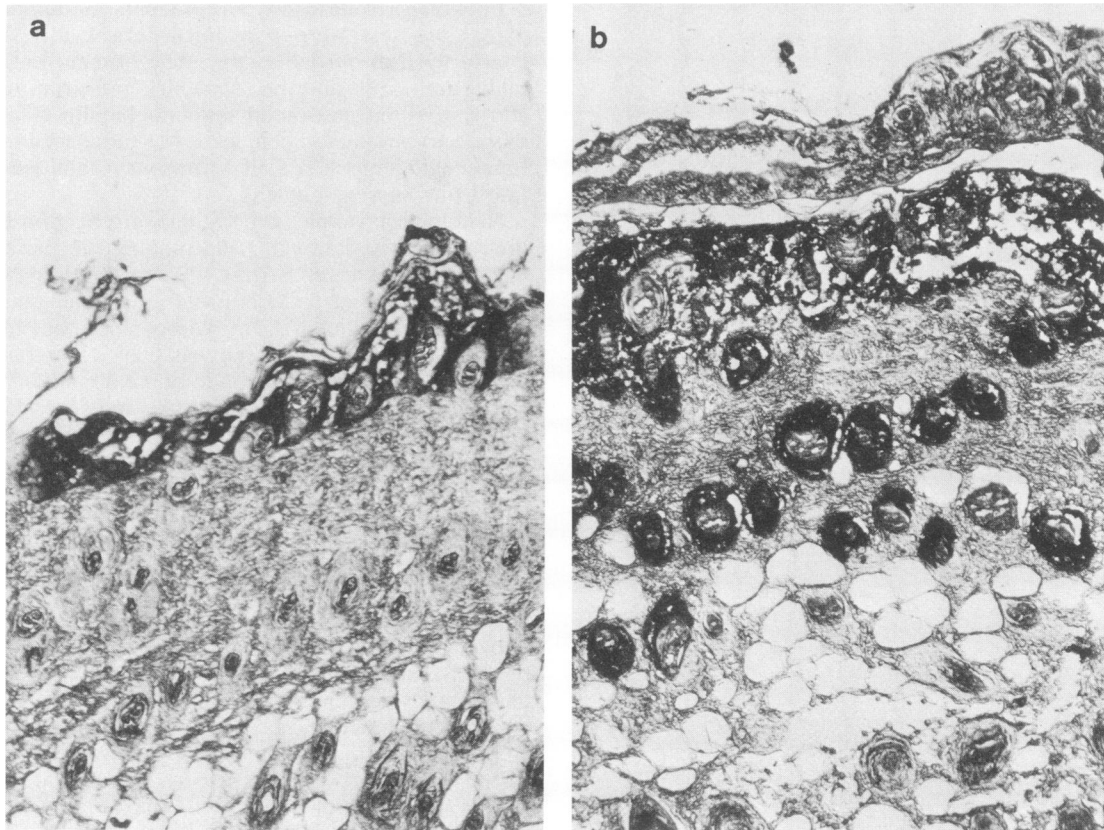
FIG. 3. Immunoperoxidase staining for viral antigen. (a) Skin of the lower flank. The specimen was taken on day 4 p.i., which is 24 h after virus could first be detected at this site. Note the localization of virus to the epidermis. (b) More widespread distribution 24 h after infection at the inoculation site.

to the lesions previously reported and illustrated in mice (2, 12, 25). The inoculation site is identified by a scab produced during scarification of the skin. The zosteriform band extends from the posterior to the anterior midline and includes the site at which virus was initially administered. Samples of skin from the inoculation site and the lower flank were separately tested for the presence of infectious virus. In all of our experiments, inoculation of virus by scarification of the skin produced a reliable and consistent infection, with development of a zosteriform lesion in all mice allowed to survive for 4 days or more.

Figure 2 shows that virus recoverd from the site of inoculation reached a peak titer 2 days p.i. At this stage no zosteriform spread had occurred. However, virus was detectable in the lower flank by day 3 p.i. in all cases. All surviving mice developed an interrupted bandlike lesion on day 4 p.i., corresponding to the development of peak titers in the lower flank skin. The lesions were confluent by day 5.

Effect of sensory neurotomy on development of the zosteriform lesion. We investigated the effect of sensory denervation on the spread of virus to the lower flank. A total of 10 mice had their sensory nerves to the left flank transected 6 days prior to infection; control animals received a mock operation. After the midflank was infected, the animals were observed daily for the development of a zosteriform eruption. All 10 control animals developed visible rashes on day 4 p.i.; 8 progressed to severe lesions by day 6, and 6 of these mice died on or before day 10, with clinical evidence of hind limb paralysis and encephalitis. Each animal without senso-

ry innervation developed a small ulcerated lesion at the inoculation site, but none developed zosteriform rashes, and all survived.

Infectious virus could not be detected in the lower flank of any of the 10 denervated mice, which were killed and tested 4 days after infection. We intend to report separately the effect of sensory neurotomy on the local replication of virus at the inoculation site.

Histopathological studies and localization of virus by the immunoperoxidase technique. By immunoperoxidase staining viral antigen was demonstrated in the skin of the lower flank on day 3 p.i., corresponding to the appearance of infectious virus detected by the plaque assay. Figure 3a shows that virus was at first highly localized to the epidermis. At this stage, hematoxylin-eosin-stained sections from the lower flank (data not shown) showed hyperplasia and abnormality of epidermal cells in infected regions, but little or no inflammatory response. This contrasts with the early cellular infiltrate that was seen at the inoculation site; samples from mice killed at two hourly intervals after infection showed that cellular infiltration commenced within a few hours, in response to the trauma of inoculation and perhaps also to the growth of virus in the dermis, which is shown by immunoperoxidase staining in Fig. 3b.

Effect of adoptive transfer of immune lymph node cells on the recovery of virus from the inoculation site. Recovery of virus from the inoculation site is described above (Fig. 2). In the same experiment a separate group of 15 infected mice received immune cells intravenously 24 h p.i.; 7-day draining
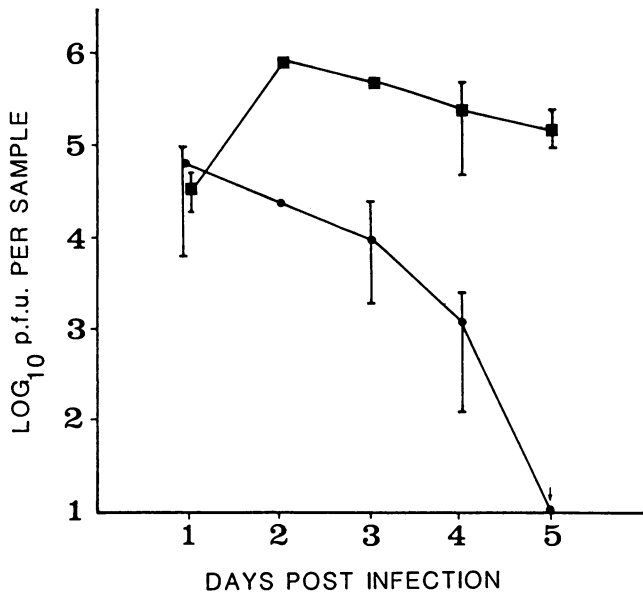
FIG. 4. Effect of immune cells given 24 h p.i. on the titer of infectious virus recovered from the inoculation site. The control animals (■) received no cells. Each test animal (●) received 2×10^7 7-day draining lymph node cells intravenously. Each point represents the arithmetic mean of values obtained from a group of three mice. Bars indicate ranges of values, where variation occurred.
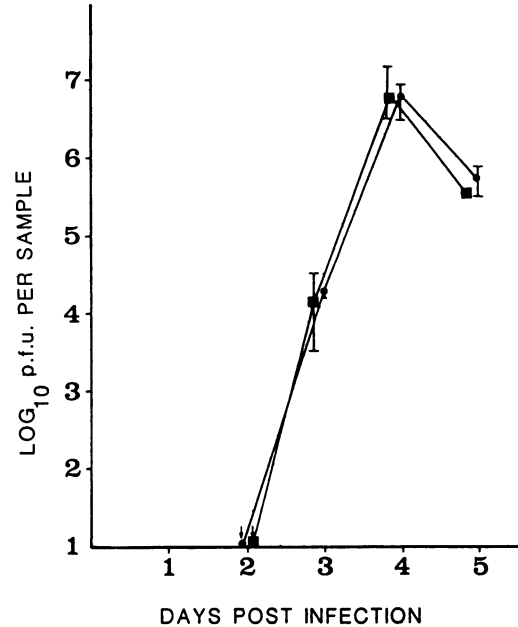
FIG. 6. Effect of 2×10^7 draining lymph node cells given 48 h p.i. (■) on recovery of infectious virus from the lower flank, compared with controls receiving no cells (●). Each point represents the arithmetic mean of values obtained from a group of three mice. Bars indicate ranges of values, where variation occurred.

lymph node cells were used because it is known that this population exhibits a wide range of antiviral activity in vitro (13, 17). Figure 4 shows the effect of this adoptive transfer on the virus titer at the inoculation site. These cells greatly accelerated the clearance of virus from the skin, compared with no cells. In preliminary experiments, lymph node cells taken from uninfected mice were transferred to control animals. However, this was found to make no difference to the virology of the system. Subsequently, the use of these cells was abandoned to avoid killing large numbers of normal mice. The effect of immune cells was seen 24 h after transfer,

and virus was undetectable by day 5 p.i. The skin of the lower flank was assayed separately, and the results are described below.

Effect of adoptive transfer of immune cells given 1 day p.i. on zosteriform spread of virus. Skin samples from the lower flanks of the mice used in the previous experiment were assayed to determine the effect of immune cells on viral spread through the nervous system. In addition, the sensory ganglia were tested for the presence of infectious virus and compared with unprotected controls (Fig. 5a). Infectious
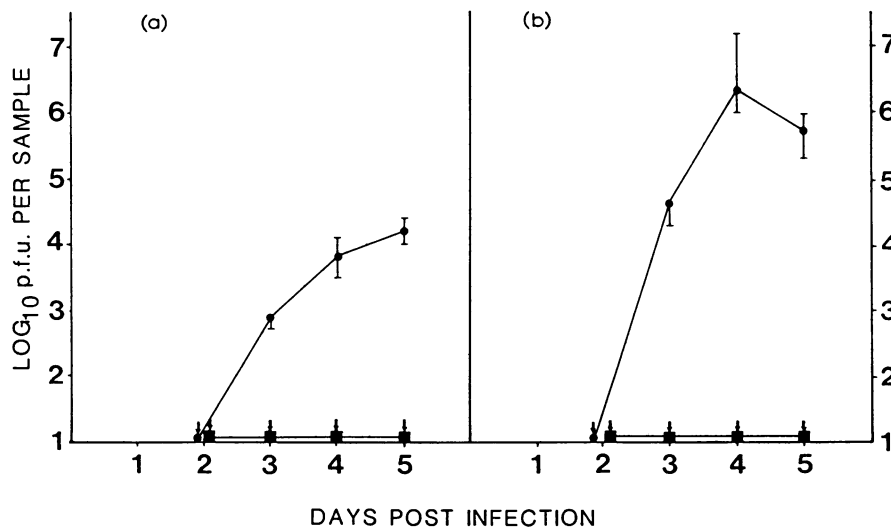

FIG. 5. Effect of 2×10^7 draining lymph node cells given 24 h p.i. (■) on the recovery of infectious virus from the dorsal root ganglia (a) and the lower flank (b), compared with controls receiving no cells (●). Each point represents the arithmetic mean of values obtained from a group of three mice. Bars indicate ranges of values, where variation occurred.

virus was consistently detected in the sensory ganglia of the control mice 3 days p.i., but not on day 2. However, in a separate experiment, 10 unprotected mice were killed 48 h p.i., and the ganglia were kept in explant culture in HEPES-buffered GMEM for 6 days before being tested; 9 of the 10 mice were positive for virus, showing that some virus had reached the ganglia within 48 h of infection in the majority of animals.

Immune cells given 1 day p.i. always led to a reduction of infectious virus in the ganglia to below the threshold of the assay used. Hence, it is not surprising that this procedure also eliminated zosteriform spread to the skin of sites remote from the inoculation (Fig. 5b). Mice allowed to survive for 4 days or more did not develop rashes, and none showed any neurological signs.

Effect of immune cells given 2 days p.i. on zosteriform spread. Completely different results were obtained when the adoptive transfer of cells took place 48 h after infection (Fig. 6). Two groups of 12 mice were infected, and one group received immune cells on day 2. Three animals from each group were killed on days 2 through 5, and the lower flank skin was tested for the presence of infectious virus. In this instance zosteriform spread was not prevented by the immune cells, and more surprisingly the growth of virus in the skin was not subsequently modified by the presence of these cells. This could not be attributed to a defect in their activity, because the cells administered were from the same pool as the cells used in the previous experiment and were given concurrently. Conventional histological examination of the skin of these mice on days 3 and 4 p.i. (the first and second days of lower flank infection) again showed a remarkable lack of cellular infiltration, despite clear histological changes in the epidermal cells characteristic of herpes simplex infection. Mononuclear cell infiltration appeared between days 4 and 5, when considerable destruction of the epidermis was also present.

Effect of T-cell depletion on the function of immune cells. Draining lymph node cells were depleted of T cells in vitro by incubation with anti-thy 1 antibody and complement. Control cells were incubated with normal mouse serum and complement. The surviving cells (64% of test cells and 94% of control cells) were transferred to recipients 1 day after infection. The mice were killed on day 5 p.i., and the lower flank skin was tested for the presence of infectious virus. The $\log_{10}$ virus titers for the mice given cells treated with anti-thy 1 antibody and complement were 5.8, 5.9, 5.9, 6.1, and 6.2; the titers for the five mice given cells treated with complement alone were all less than 1. T-cell depletion eliminated the protective effect of the immune cells.

DISCUSSION

The emergence of virus from nerve endings and the subsequent infection of epidermal cells, as occurs in the zosteriform reaction, provide a powerful model with which to study recrudescent lesions. In the system which we describe here, virus is detected in the epidermis distant from the inoculation site 3 days after infection, and sectioning of the appropriate sensory nerves prevents this, thus confirming that virus returns from the nervous system to the skin of the flank between 48 and 72 h after infection. As zosteriform spread was not found until day 3, it follows that the potent antiviral action of immune lymph node cells given 1 day after infection reflects a wholly peripheral action; i.e., the cells act directly on herpes-infected skin. That this effect is T cell dependent is in accord with previous findings from this (8, 13) and other laboratories (11). However, data from the

present study indicate that the zosteriform reaction may be an important component of all primary cutaneous herpes infections and that this should be considered when mechanisms of immunological protection are investigated.

The role of T cells in recovery from acute HSV infection has been documented (3, 10–13, 15, 16, 19, 21). However, the mechanisms by which immune cells exert their effects are, as yet, not fully understood. It is known that macrophages are recruited into areas of acute viral infection by lymphokine-releasing T cells (1). This process is a central feature of delayed-type hypersensitivity, which has been extensively characterized with respect to HSV and has been shown to play an important part in the clearance of virus from peripheral lesions during acute infection in mice (13, 14). Cell-mediated anti-HSV activity may also include a cytotoxic T-cell component (16, 17, 20, 23), although a role for cytotoxic cells in vivo is difficult to demonstrate (A. A. Nash, K.-N. Leung, and P. Wildy, in B. Roizman, ed., The Herpesviruses, vol. 4, in press).

T cells may also cooperate with B cells for the production of anti-HSV antibodies. There have been a number of studies on the action of antibodies in preventing the spread of a virus to the nervous system and in protecting against lethal infections (4, 7, 8, 12). However, such studies offer little insight into the mechanisms limiting the appearance of virus in the skin, such as occurs during zosteriform spread or true recrudescence. The effect of antibody in this situation has still to be critically evaluated, but preliminary evidence from our laboratory indicates that the passive administration of high-titer neutralizing antibody can interrupt zosteriform spread at the sensory nerve-epithelial cell junction. However, neither antibody administered at 66 h (unpublished data) nor immune lymphoid cells transferred at 48 h after initial infection prevented zosteriform spread; nor did the immune cells affect the titer of virus recovered from the lower flank during the first 3 days of infection at this site.

The reason for this apparent failure of immunity is not clear. It could be that the transferred immune cells are diverted to sites other than those associated with the zosteriform lesion, hence diluting their effectiveness. Alternatively, immune cells may fail to recognize virus as it first appears in the epidermis. Support for the latter possibility is found when histological sections of the early lesion are studied. Virus is localized to the epidermis for at least 24 h after it emerges from the nerves, and there is little cellular infiltration until a clinical lesion occurs. Consequently, following infection of the epidermis in recrudescence, cellular immune mechanisms may be rendered ineffective because of lack of recruitment, until such a time as viral cytolysis has caused a breakdown of the epidermal basement membrane and subsequent dermal damage, constituting the clinical lesion. This observation may help to explain the paradoxical occurrence of recrudescent lesions in humans in the face of active immunity (24).

In conclusion, we report here a mouse flank model for the final step in the development of a recrudescent lesion (i.e., the infection of epidermal cells) and show its usefulness in the investigation of immune processes that are likely to be of value in modifying the appearance of a clinical lesion after reactivation. It is evident that effector mechanisms that have access to areas of primary infection are not necessarily available to combat recurrent disease.

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