

Vaccinia Virus Meningitis in Mice After Intracerebral Inoculation

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The pathogenesis of experimental vaccinia virus infection in weanling mice after intracerebral inoculation was followed with virological, histological, and immunohistological methods. High-dose inoculation resulted in early mortality. After low-dose inoculation, virus spread from brain to thoracic and abdominal viscera probably by an undetected early viremia. Virus did rise to detectable levels in blood by day 5 and was found to be associated with the mononuclear cell fraction. By day 12, 30% of the animals had died and no further deaths occurred. Rise of neutralizing antibody correlated with disappearance of cell-free virus in blood, brain, and viscera. Virus was present in the brains of animals for 20 days after inoculation. This animal model may be useful to study mechanisms of persistent central nervous system virus disease relevant to man.

Postvaccinal encephalomyelitis was first described by Bastiannse in 1924 (1). Central nervous system (CNS) complications of smallpox vaccination, although infrequent, have been well-documented in recent years (18). Current ideas surrounding postvaccinal encephalomyelitis have supported an immune-mediated pathogenesis, which may have a counterpart in experimental allergic encephalomyelitis of animals (19). The report of vaccinia virus isolation from children with postvaccinal encephalitis, as late as 35 days after inoculation, challenges the validity of a purely allergic pathogenesis and suggests virus participation in postvaccinal CNS disease (9).

Among previous experimental vaccinia studies, Cassel (5) showed that vaccinia virus could produce fatal encephalitis after intracerebral inoculation of mice. Briody (4), studying ectromelia and vaccinia infections, noted a strain-specific CNS susceptibility in BAGG mice after extraneural inoculation. The neurovirulence of various strains of vaccinia virus has recently been reviewed (23). The role of immunity in the pathogenesis of vaccinia virus infection has also been studied (7, 12, 25), and results suggest that both cell-mediated and humoral mechanisms function in the recovery from systemic infection; however, the role of humoral immunity was not characterized for target organ infection. Immunosuppression has generally not potentiated primary virus infection when the agent is introduced directly into the target organs (8, 11, 16, 17).

Few studies have been concerned with the pathogenesis of acute cerebral vaccinia infection, and none has reported the persistence of virus in the CNS of surviving animals. In view of the demonstrated, but poorly understood, relationship between vaccinia virus and CNS disease, a laboratory animal model demonstrating the presence of vaccinia virus in brain might offer insights into the cerebral events occurring in the human disease postvaccinal encephalomyelitis. This study characterizes the pathogenesis of acute vaccinia in mice and further shows that vaccinia virus may persist in brain for up to 20 days in the presence of significant levels of neutralizing antibody to the agent.

MATERIALS AND METHODS

The IHD strain of vaccinia virus, obtained from the American Type Culture Collection, had been passaged 51 times in mouse brains, 4 times in chicken embryos, 4 times in LLC-MK₂ tissue cultures, and 1 time in chicken embryo tissue culture. Stock virus, prepared from the supernatant fluid of BSC-1 tissue culture (Grivet monkey kidney) contained $10^{6.3}$ mean lethal doses per 0.03 ml when titrated by intracerebral (i.c.) inoculation of weanling mice and $10^{5.3}$ mean tissue culture infective doses (TCID₅₀) per 0.1 ml when titrated in monolayer culture of BSC-1 cells.

A pilot study employed intraperitoneal and intravenous routes of inoculation of inbred BALB/c white mice; however, virus could not be recovered from brain. Thus, an i.c. route of inoculation was used for further studies. Three groups of inbred BALB/c white mice, 2 to 3 weeks old, received a single i.c. inoculation containing either 10^6 or 10 mean lethal

doses of virus contained in 0.03 ml of Hanks basic salts solution (HBSS) and were collected at varying periods from 1 to 31 days after inoculation. All surviving animals were observed for recrudescence of symptoms or new signs of illness for at least 3 months.

Virus content was assayed in blood, brain, and thoracic and abdominal viscera by pooling specimens of each from four animals. Thoracic viscera consisted of heart and lung, whereas abdominal viscera contained liver, kidney, and spleen. Blood was hemolyzed by freezing and thawing. Solid organs were prepared as 10% (wt/vol) suspensions in HBSS by homogenization and were stored frozen. Ten-fold dilutions were made in HBSS, and titrations were performed in duplicate on confluent BSC-1 monolayers grown in multiple 2-cm² well plates (Falcon Plastics). Cultures were incubated at 37 C and examined for cytopathic effect each day for at least 10 days. Titers were expressed as TCID₅₀ per gram of tissue or milliliter of blood (20).

When virus could no longer be recovered from homogenized tissues, brains and thoracic and abdominal viscera were harvested from four animals, washed in HBSS, minced, trypsinized for 20 min, passed through gauze mesh, and then centrifuged at 1,500 rpm for 10 min. The cells were washed twice in HBSS, suspended in 1 ml of Leibovitz (L15) medium with 5% fetal calf serum, and co-cultivated with BSC-1 cell monolayers in 25-cm² flasks (Falcon Plastics). The cultures were incubated at 37 C and examined for cytopathic effects for at least 10 days.

The viremia was evaluated by pooling the blood from five animals and separating components using a Ficoll-Hypaque gradient (3). Serum, leukocytes, and erythrocytes were then assayed separately for virus content by co-cultivation with BSC-1 cell monolayers in flasks. Cultures were incubated at 37 C and examined for cytopathic effects for at least 10 days.

Tissues for conventional histological study were preserved by immersion in Bouin fixative, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Fluorescent-antibody staining employed a direct fluorescein conjugate (1:40 dilution) prepared from vaccinia hyperimmune rabbit serum and supplied by Edwin H. Lennette, Berkeley, Calif. Fresh abdominal and thoracic viscera and coronally sectioned brains were frozen within 3 min of removal, stored at -70 C, and sectioned at 10 μm in a cryostat. Sections were dried, fixed in acetone for 10 min, overlaid with the fluorescein conjugate for 30 min, washed twice in phosphate-buffered saline, and mounted in neutral glycerol. Controls, employing homotypic-blocking antisera and uninfected tissues, were processed in a similar manner. Brain tissue was examined for the presence of antigen sequentially for 31 days after inoculation in the manner described above.

Positive immunofluorescence in brain was confirmed with an indirect immunoperoxidase, light microscopy technique, derived by modification of a previously described direct staining method (22). Sections were dried, fixed in acetone for 10 min, and

overlaid with vaccinia hyperimmune rabbit serum for 20 min. Sections were washed twice in phosphate-buffered saline and overlaid with peroxidase-conjugated anti-rabbit globulin (Miles Laboratories, Elkhart, Ind.) for 20 min. After two washes in phosphate-buffered saline the sections were overlaid with 0.05% diaminobenzidine and 1% peroxide for 10 min, stained with toluidine blue, dehydrated in acetone and xylene, and mounted in Permount for examination by light microscopy.

Trypsinized viscera and brain cells from late-surviving animals, co-cultivated with BSC-1 cell monolayers, were checked for the presence of viral antigen, regardless of the development of cytopathic effects, in the following manner. After 10 days of incubation at 37 C, cells were scraped from the flasks, centrifuged at 1,500 rpm for 10 min, washed twice in HBSS, and suspended in 1 ml of normal saline. A single drop was placed on a microscope slide and allowed to air dry for 1 h. The cells were then fixed in acetone for 10 min and stained with the direct fluorescein conjugate as described previously. Positive- and negative-control preparations were always included.

Serology was performed on sera pooled from four animals and stored at -70 C. Assays included a plaque reduction neutralization technique and hemagglutination inhibition (6) using a microtiter modification (21).

RESULTS

After i.c. inoculation of 10⁶ TCID₅₀ of virus (high dose), weanling mice appeared well until day 2, when the majority became lethargic, stopped feeding, and developed ruffled fur. By the end of 5 days, all had died (Fig. 1). Death was often preceded by tonic seizures.

When an i.c. inoculum of 10 TCID₅₀ of virus (low dose) was used, the weanling mice appeared well until approximately 6 days, when the majority became lethargic, stopped feeding, and developed ruffled fur. Between 6 and 12 days, there was a 30% mortality, and death was, at times, preceded by tonic seizures (Fig. 1). The animals that recovered and were observed over a 3-month period showed no recrudescence of symptoms seen at 6 days, and paralysis was never evident. Animals showing little evidence of illness even during the acute phase did not become sick at a later date. Feeding, mating, and motor activity appeared normal in long-term survivors.

Determination of virus content after i.c. inoculation with the high dose showed that infection was not limited to the brain although the CNS was the major site of virus replication (Fig. 2). Study of virus replication in this group of animals was limited because of the early high mortality. Viremia, however, was first noted on day 2 after rising titers were observed in other organs.

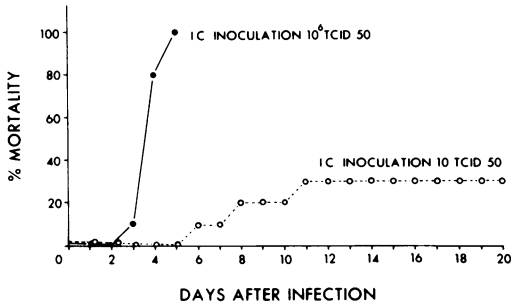


FIG. 1. Comparison of mortality in BALB/c weanling mice receiving either high-dose or low-dose i.c. vaccinia inoculum.

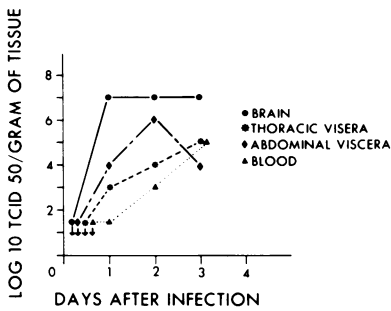


FIG. 2. Growth curves of vaccinia-IHD in weanling mice after high-dose i.c. inoculation. The first point of virus titration was at 12 h after inoculation when no virus was detected by homogenization and titration in any of the sampled tissues. Less than 2 logs of virus per gram of tissue could not be identified by this assay.

Determination of virus content after i.c. inoculation with the low dose permitted a comprehensive evaluation of virus replication (Fig. 3). Virus appeared first in high titer in brain. Less virus was isolated at a later time from thoracic and abdominal viscera. Viremia could not be detected until day 6. Separation and assay of blood components revealed that virus was carried exclusively in the mononuclear cell fraction. By day 7, titers began to fall in all organs, and no free virus was detectable by day 12. Attempts by homogenization and freeze-thaw to find virus in brain on days 12, 14, 16, 18, and 20 were unrevealing. Nevertheless, virus was consistently recovered from brain by co-cultivation through day 20 but not on day 31.

Gross brain morphology in both high- and low-dose animal groups appeared normal. Histological sections of brain and abdominal and thoracic viscera of animals given a high dose of virus showed only a mild meningitis and a mild interstitial pneumonitis prior to death (Fig. 4). In contrast, the low-dose group had developed a pronounced meningeal reaction by day 5, which

was not evident in surviving animals at day 12 (Fig. 5). No histological changes were found in other organs. Thus, although all animals contained large quantities of virus in several organs (Fig. 2 and 3), histological evidence of disease was scant, except for marked meningeal pathology at day 5 in animals that received a low dose of virus.

Fluorescent-antibody technique revealed the presence of antigen in meningeal, choroid, and ependymal cell cytoplasm by 48 h (Fig. 6) and was confirmed by immunoperoxidase, light microscopy. The involvement of the CNS was shown by these methods to be more extensive than was suggested by routine pathology. Fluorescence in meninges, choroid, and ependyma was maximal by 7 days. It was granular in character and exclusively cytoplasmic in location. During the 12-day period, when virus was readily recovered from brain by homogenization and the freeze-thaw technique, antigen was consistently demonstrable in the stated location with a 1:40 conjugate dilution. During the persistent period, between 12 and 20 days when co-cultivation was necessary to demonstrate virus in brain, antigen persisted in meningeal and ependymal cell cytoplasm. Despite consistent localization, fluorescent brightness diminished after day 12, and a reduction in conjugate dilution was necessary to demonstrate brain antigen at day 18. By day 31, antigen was no longer demonstrable. Antigen was never detectable in brain parenchyma. In CPE-positive cultures co-cultivated with brain cells from late-surviving animals, marked cytoplasmic fluorescence of individual cells was noted. Cultures without CPE did not exhibit specific fluorescence.

Neutralizing antibody was first detected at day 9 and rose through day 20. Its rise coincided with fall-off of free virus in brain, blood, and other organs. Hemagglutinin-inhibiting anti-

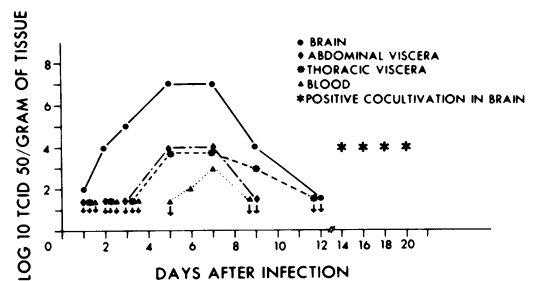


FIG. 3. Growth curves of vaccinia-IHD in weanling mice after low-dose i.c. inoculation. Note the isolation of cell-associated virus late in the infection. Less than 2 logs of virus per gram of tissue could not be identified by this assay.

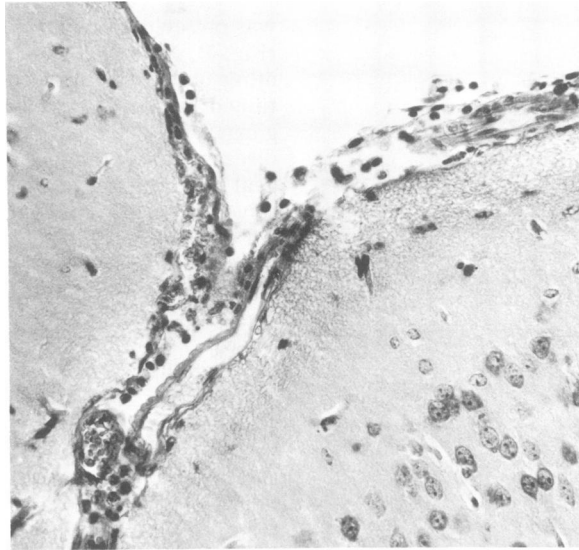


FIG. 4. Medial frontal cortex 72 h after high-dose *i.c.* inoculation of vaccinia-IHD showing a mild inflammatory meningeal reaction (hematoxylin and eosin, $\times 270$).

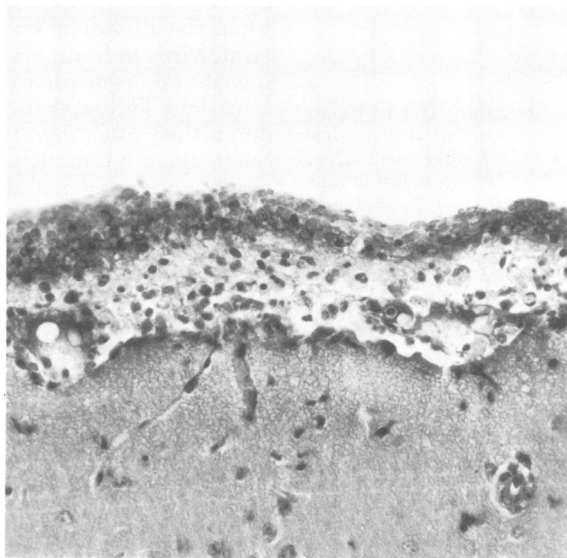


FIG. 5. Parietal cortex 120 h after low-dose *i.c.* inoculation of vaccinia-IHD showing a pronounced inflammatory meningeal reaction with invasion of the Virchow-Robin space (hematoxylin and eosin, $\times 170$).

body rose early during the course of infection and began to fall by day 14. It was present in higher titer than neutralizing antibody but did not appear to correlate with the fall in viral titers in viscera and brain (Fig. 7).

DISCUSSION

An attempt has been made to study the pathogenesis of infection after IC vaccinia virus inoculation of weanling inbred mice. Although

early work by Briody (4) suggested that certain strains of mice would develop CNS infection after parenteral inoculation, a pilot study employing BALB/c weanlings (historically, genetic derivatives of BAGG mice), the strain used in his experiments, failed to show CNS viral invasion after extraneural inoculation. Therefore, an *i.c.* route of infection was used. It soon became apparent that survival, viral replication, severity of pathology, and viral persist-

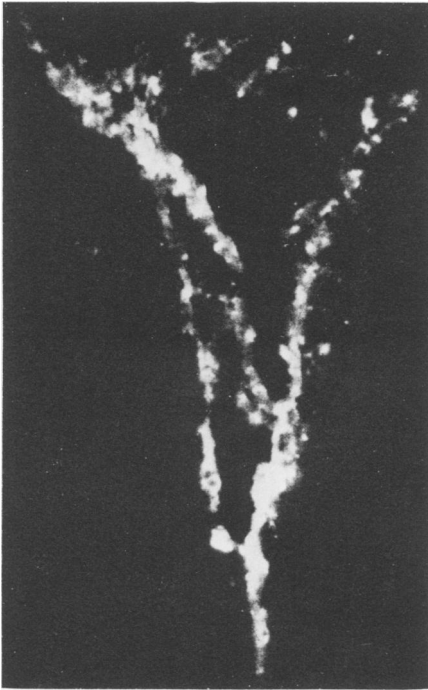


FIG. 6. Section of brain 48 h after high-dose i.c. inoculation of vaccinia-IHD showing vaccinia antigen in ependymal and choroid cells lining the third ventricle (fluorescent-antibody stain, $\times 140$).

ence were dose related. Inoculation of 10^6 TCID₅₀ of virus resulted in early viremia, rapid dissemination and virus growth in thoracic and abdominal viscera, disproportionately little pathology, and high, early mortality. When only 10 TCID₅₀ were used, viral replication in brain was slower and cerebral pathology was striking. Despite early spread to thoracic and abdominal viscera, $\frac{2}{3}$ of the group survived. The day-5 onset of viremia in this group may be explained by circulatory spread after CNS replication or by early viremia from spillover of virus inocula, with successive replication in abdominal and thoracic viscera and subsequent hematological seeding.

The dilemma surrounding the relationship between dose of virus, mortality, and histopathological change needs clarification. Mortality correlated directly with the amount of virus inoculated, whereas histological evidence of infection correlated inversely with strength of the inoculum. This could be explained simply on the basis that increased survival in the low-dose group allowed time for the development of a marked inflammatory response. If so, what caused the high, early mortality in the absence of marked histopathology in the high-dose

group? Recent work by Burks et al. (J. S. Burks, H. F. McFarland, and O. Narayan, *Neurology* 25:350, 1975) with Newcastle disease virus has demonstrated that clinical disease and mortality may occur in BALB/c mice when only trace amounts of infectious virus are present and in the absence of CNS pathology. They postulated that suppression of neuronal metabolic activity by the virus produced death. Virus-induced suppression of CNS cellular metabolism remains a possible explanation for the incongruity between pathology and mortality in the present study. The restriction of viral antigen to choroid, ependymal, and meningeal cells also suggests that an indirect metabolic influence on CNS parenchyma may be operational.

The persistence of cell-associated virus in brain 20 days after inoculation in this study is noteworthy. The appearance of serum-neutralizing antibody correlated with the decline in extracellular virus in both brain and viscera. The virus appeared to become altered so it no longer exerted an obvious deleterious effect on the host but persisted in a cell-associated state. A true alteration in growth characteristics of the agent is suggested by the mandatory use of co-cultivation for viral recovery; however, this phenomenon may merely indicate the necessity of using this sensitive technique in retrieving small amounts of persistent, unaltered, intracellular virus. No direct evidence, such as electron microscope visualization of virions, is available to demonstrate an intracellular location of persistent brain virus. This location is implied by repeated failure to find virus in brain on and after day 12 using techniques that would demonstrate more than 2 logs of extracellular virus per gram of tissue.

A recent review of the cell surface suggests that the topography of membrane proteins can be modified by exposure of cells to various substances (2). Viral replication dependent upon budding from the cytoplasmic membrane might

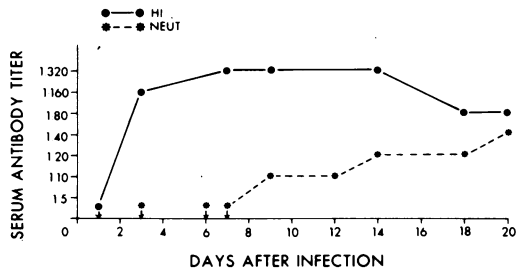


FIG. 7. Serum antibody response to vaccinia antigens in weanling mice inoculated i.c. with vaccinia virus on day zero.

be influenced by the action of antibody on receptor sites. Alternatively, poxviruses, which for the most part acquire their envelopes through de novo synthesis, are recognized as being able to change membrane properties (15). The mobile characteristics of membrane structures may provide a potential for antibody to interact with the agent and alter the pathogenesis of disease at the cellular level. Similar findings with experimental measles (13) and herpes (24) infections also suggest that the disappearance of cell-free virus in vivo is related to the development of neutralizing antibody. Thus, the humoral immune response may alter the infectious characteristics of several viruses, resulting in continued presence of viral constituents in host organs, which could, in turn, contribute to disease processes not clearly definable as infections.

Postvaccinal encephalomyelitis is an acute, monophasic CNS disease of man, characterized pathologically by inflammation and perivascular demyelination. What constitutes the "trigger" in human cases of postvaccinal encephalomyelitis, including those complicating vaccinia immunization, remains an unanswered question. Antigen induction, i.e., the formation of haptens, by the incorporation of viral components into host CNS cells and membranes, resulting in an anti-CNS immune response, might be related to the prolonged presence of viral components in the CNS (10). The recovery of vaccinia virus from several cases of postvaccinal encephalomyelitis (9) gives credence to the possible association between persistent CNS virus and postvaccinal pathology. The demonstration in the present study of CNS vaccinia virus well beyond the initiation of the host immune response and beyond the period when postvaccinal encephalomyelitis usually occurs in man is of significance. Future studies of this model, manipulating the immune recognition and response to viral antigen, may reveal the nature of viral participation in postvaccinal encephalomyelitis.

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