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# Vaccinia Virus Infection of the Central Nervous System in X-Irradiated Mice

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The effect of X-irradiation on experimental vaccinia infection of BALB/c mice was studied. As compared with nonirradiated controls, the X-irradiated animals exhibited (i) a time lag in virus replication (delayed, but protracted replication); (ii) a delayed and repressed immune response; (iii) more severe acute cytocidal infection of leptomeninges, choroid plexus, ependyma, and vessels, with extensive damage to the brain-barrier system; and (iv) noncytocidal, latent infection of glial cells and neurons. Several animals developed acute or subacute demyelination disease, resembling experimental allergic encephalomyelitis or postinfectious encephalomyelitis.

Experimental vaccinia infection was employed in previous experiments as a model of virus-induced demyelination (7, 10, 11, 13, 14). Vaccinia virus was chosen because of its well-established etiological role in human postvaccinal encephalomyelitis. Postvaccinal and postinfectious encephalomyelitis are typical examples of demyelination disease. Data obtained thus far indicate that animals with a competent immune system develop an acute, lytic choriomeningitis, ependymitis, and vasculitis. The severity of the infection depends on the virulence and the dose of the virus, route of inoculation, and species and age of the animals (11). Transient demyelination and autoimmunity appear in some severely affected animals. However, no typical postvaccinal encephalomyelitis develops. The present study deals with the vaccinia infection of BALB/c mice which had been made immunologically incompetent by X-irradiation. The question under consideration was whether a transient immune deficiency enhances the expression of postvaccinal encephalomyelitis.

## MATERIALS AND METHODS

**Animals.** All studies were performed in female BALB/c mice (Central Institute for Laboratory Animals, Hannover, W. Germany) weighing 10 to 12 g and kept under standard conditions. The experimental protocol is given in Table 1.

The Elstree strain of vaccinia virus was grown on chicken embryo fibroblasts, purified through differential centrifugation, and suspended in McIlvain buffer. The infectious titer, determined in HeLa cells, was $10^7$ plaque-forming units per ml. The infection was induced by an intracerebral injection of 0.03 ml of virus suspension containing $10^4$ plaque-forming units per ml.

**Irradiation.** Whole-body irradiation was performed 24 h before infection. A Müller MG300 unit (C. H. F. Müller, Hamburg, W. Germany) was employed with the following specifications: 250 kV; 12 mA; 0.7-mm Cu filter; dose rate, 53/min; distance from focus to animal, 40 cm. All animals received 300 rads. Previous studies (unpublished) had shown that this dosage is immunosuppressive in mice, but does not kill the animals.

**Virological studies.** Beginning on day 3 after infection, two animals were sacrificed daily for 14 days for infectivity assay. The brains were removed under sterile conditions and stored at −20°C. After all of the test material had been collected, the brains were thawed and homogenized in phosphate-buffered saline. Serial 10-fold dilutions were made in phosphate-buffered saline, and 0.1-ml portions of each dilution were added to the growth medium of a monolayer of HeLa cells in 50-ml plastic bottles (Nunc, Inc., Copenhagen). After 2 h the supernatant fluid was removed, the cells were rinsed three times with phosphate-buffered saline, and fresh maintenance medium was added. The cultures were examined for cytopathic effects after 48 to 96 h. If no cytopathic effects were observed, serial passages were done. If there was no evidence of virus growth after two passages, the culture was considered negative.

Serological studies were performed on sera pooled from three to four animals on days 4, 7, 18, and 60. The assays included the following.

**(i) Plaque reduction neutralization technique.** The plaque reduction neutralization technique was performed by the method of Downie and Kemp (4).

**(ii) Complement-dependent gliotoxic serum activity using two cell types as a target.** Rat glial tumor cells CCL $107/C_6$ (Flow Laboratories, Bonn, W. Germany) and primary cultures from the spinal cord of 14- to 17-day-old rat embryos (each $10^6$/ml in Dulbecco minimal essential medium plus 10% fetal calf serum) were cultured at 10 μl per well in microtiter plates. After incubation for 24 h (37°C, 5% $CO_2$), 100 μl of the serum samples (heat inactivated at 56°C for 30 min and serially diluted 1:2) was added to each well and incubated for 30 min. Then 10 μl of a previously determined optimal dilution of guinea pig complement

TABLE 1. *Experimental protocol and clinical signs*

| Treatment | No. of mice | No. of mice with: | | |
|---|---|---|---|---|
| | | General signs | Paresis | Death |
| X-irradiation + intracerebral infection | 40 | 40 | 7 | 11 |
| Intracerebral infection (control A) | 40 | 32 | 0 | 0 |
| X-irradiation (control B) | 10 | 7 | 0 | 0 |

(Behringwerke, Marburg, W. Germany) was added and incubated for an additional 90 min. As a positive control, rabbit anti-mouse glial cell membrane antiserum was produced by immunizing rabbits with 30 mg of lyophilized mouse glial cell membrane in 300 μl of 0.15 M NaCl plus 300 μl of incomplete Freund adjuvant injected subcutaneously, followed by three booster injections. Fetal calf serum was used as a negative control. All tests were performed in duplicate, and the percentage of damaged cells was determined morphologically. The serum dilution producing 50% cytotoxicity was taken as the cytotoxic titer.

**(iii) Brain antigen antibodies.** Antibodies with an affinity for various brain antigens were estimated by indirect immunofluorescence using pooled sera diluted 1:8. Spinal cord sections from the normal rabbit served as antigen. The details of the method have been described elsewhere (13).

**Histology.** The central nervous system was examined on days 4, 7, 18, and 60. Five blocks of brain and two blocks of spinal cord from each animal were embedded in Paraplast (Sherwood, St. Louis, Mo.) and stained with the acridine orange, hematoxylin-eosin, azan, Nissl, and Kelemen methods.

**Immunofluorescence.** Both direct and indirect techniques were employed, as described in previous papers (13, 14). Unfixed and acetone-fixed sections were used, enabling the detection of both membrane and intracellular antigens. An indirect technique was used to examine viral antigens (anti-vaccinia serum from calf plus anti-bovine immunoglobulin G from rabbit coupled with fluorescein isothiocyanate) and antigenic changes in myelin sheaths (anti-basic protein antiserum from rabbit plus goat anti-rabbit immunoglobulin G, coupled with fluorescein isothiocyanate [Mallinckrodt, Tilburg, W. Germany]). A direct technique using a marked anti-mouse immunoglobulin G from rabbit was employed for examination of the permeability of the brain-barrier system. In addition, the permeability of the vessels was studied with another technique, using a 5% solution of dextran, molecular weight 150,000 (Pharmacia, Uppsala, Sweden), coupled with fluorescein isothiocyanate. Ten animals (five irradiated and five controls) were injected with 0.5 ml of solution intracardially 5 min before killing. Formalin-fixed sections showed a bright fluorescence of the vessel system (see Fig. 4).

## RESULTS

**Clinical course.** The irradiated infected mice appeared well until days 4 to 5. Then they became lethargic, developed ruffled hair, and showed loss of appetite. Seven animals developed paresis of hind limbs between days 14 and 21. Eleven animals got sick and died. The other animals recovered slowly over a period of 4 to 18 days. Some of the nonirradiated infected controls (control A) showed slight general signs, as did the irradiated noninfected controls (control B) (Table 1).

Virological findings are given in Fig. 1. In the irradiated animals the virus appeared on day 4 and reached a maximum titer on day 7. No virus was found on day 13. In the nonirradiated controls (A) the virus was detected on day 3, reaching a maximum on day 5. No virus could be

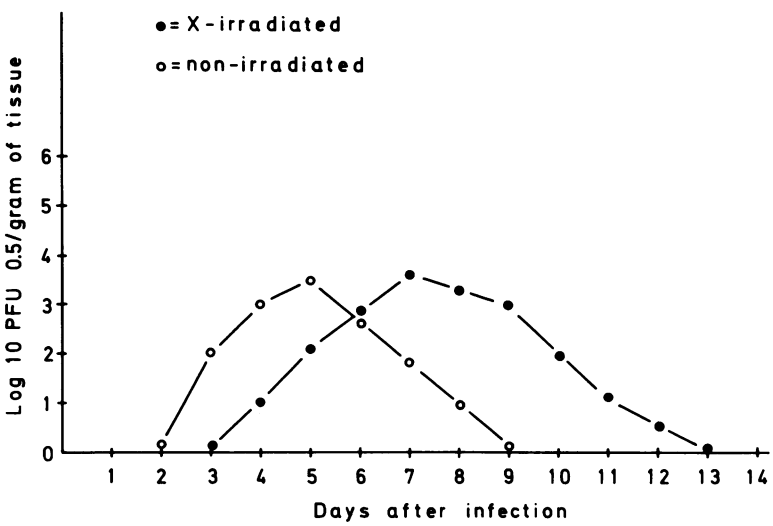



FIG. 1. *Growth curves of vaccinia virus in X-irradiated mice and nonirradiated controls (A).*

detected on day 9. The peak titers in both groups did not differ significantly.

**Serological findings.** In the irradiated mice, neutralizing antibodies were first detected on day 18, and the peak titer was 1:64. The nonirradiated controls (control A) answered with antibody production on day 12 and reached a titer of 1:512.

Gliotoxic antibodies were detected in both X-irradiated and nonirradiated infected groups (Fig. 2). They appeared later in the irradiated animals but then reached a higher titer in that group than in the nonirradiated group (control A).

Antibodies detected by immunofluorescence exhibited affinity for the glial cells. They were expressed only in the irradiated animals. A faint fluorescence of myelin sheaths was present in both groups. Irradiated noninfected animals (control B) showed no antibodies.

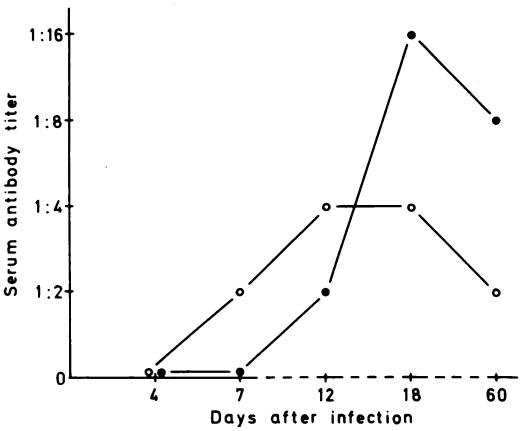



FIG. 2. *Complement-dependent gliotoxic antibodies.* (●) *X-irradiated mice;* (○) *nonirradiated mice.*

**Histology and immunofluorescence.** Irradiated, infected animals as well as nonirradiated controls (A) developed an acute choriomeningitis, ependymitis, and vasculitis with disseminated mononuclear infiltrates. Numerous cells of the leptomeninges, choroid plexus, ependyma, and vessel walls and some inflammatory cells showed degeneration or lysis. Most of these cells exhibited a bright cytoplasmic fluorescence if an anti-vaccinia serum was used (Fig. 3).

Multiple damage to cells of the brain-barrier system led to leakage of serum proteins into perivascular, subpial, and subependymal spaces or, in severe cases, into adjacent neuroparenchyma (Fig. 4B and C). In the latter cases, edema and lesions in adjacent neuroparenchyma were detectable, the most striking ones being the activation of astroglia and oligodendroglia. In myelin sheaths, antigenic changes were often encountered, apparent as irregular abolition of myelin fluorescence (indirect technique, using anti-basic protein serum). Occasionally, small focal demyelinations were observable.

Pathological conditions in irradiated animals differed in the following points. (i) The infection was delayed and protracted (Fig. 5). (ii) The lesions of the vessels and neural membranes were more severe, associated with extensive

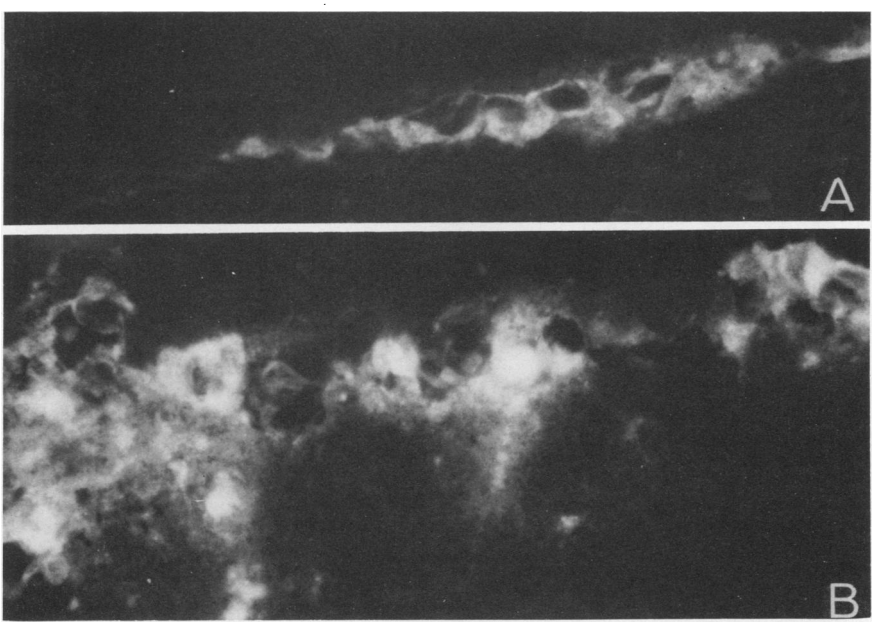
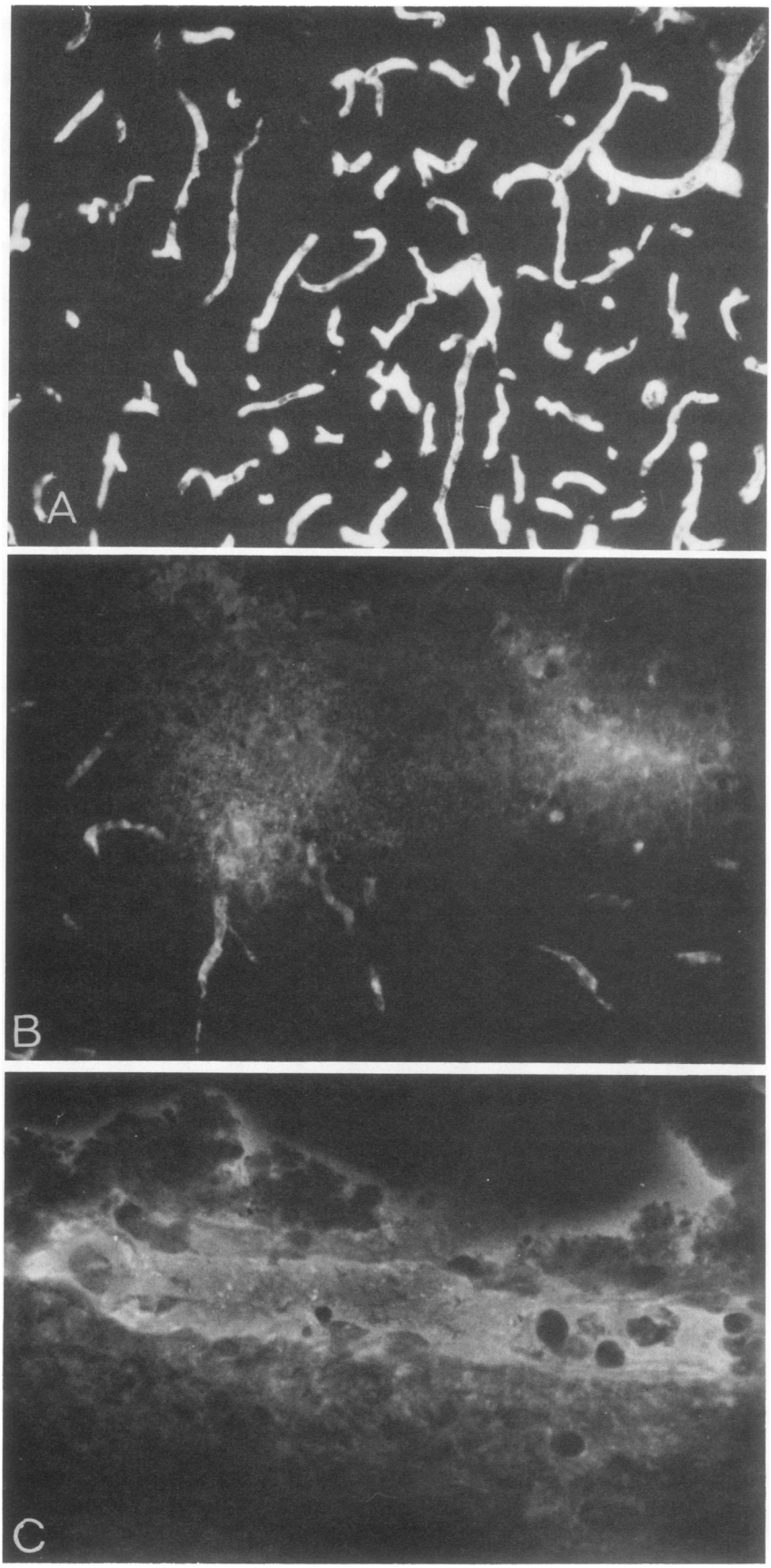



FIG. 3. *(A) Vaccinia antigen in a group of leptomeningeal cells on day 4 after infection (×500). (B) A similar finding, but with lysis of the host cells and release of viral antigen into adjacent neuroparenchyma (×500).*


A
B
C

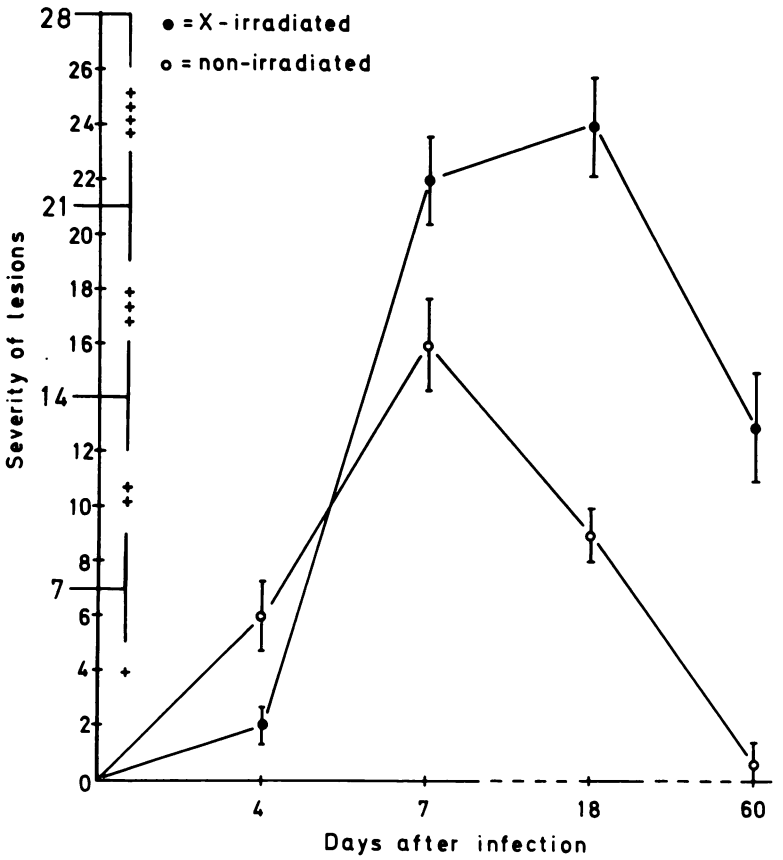



FIG. 5. *Severity of lesions in vaccinia-infected X-irradiated animals and nonirradiated controls (A). Lesions were graded as absent (0), very mild (1), mild (2), moderate (3), or severe (4), depending on inflammatory infiltrates and degenerative changes. The maximum lesion severity score possible per animal was 28 (seven sections, the maximum score per section 4). Each point represents the mean score for all animals killed on that day. The bars indicate ±1 standard deviation.*

changes in adjacent neuroparenchyma. (iii) The infection spread into the nervous tissue in some animals; numerous glial cells and a few neurons showed a faint, often granular fluorescence (Fig. 6A and B). However, no damage was observable in these cells. Unfixed sections exhibited membrane fluorescence (Fig. 6C). Another striking feature was the persistence of the fluorescence on days 18 and 60 (Table 2). In contrast, the bright fluorescence in the mesodermal and ependymal cells was only short-lived. In some cases, interstitial infiltrates appeared in the perivascular, subpial, and subependymal neuroparenchyma on day 18, 60, or both (Fig. 7). Damage of the glial cells was detectable associated with demyelination (Fig. 8), which was not detectable in the controls (A). In fact, the pathology was indistinguishable from experimental allergic encephalomyelitis. Irradiated noninfected animals (control B) sacrificed on day 7 postirradiation showed an increased permeability of the brain-barrier system. The leakage of serum proteins was diffuse and less severe than in irradiated and infected animals and affected most vessels seen in the section. Occasionally a few mononuclear cells appeared in subarachnoid or perivascular spaces, or both.

## DISCUSSION

Our findings indicate that the vaccinia virus is

FIG. 4. *(A) Fluorescence of normal vessels after intracardial inoculation of dextran coupled with fluorescein isothiocyanate (×500). (B) Slightly increased permeability of two venules with leakage of dextran in X-irradiated animal on day 4 after infection (×500). (C) Leakage of immunoglobulin G and accumulation of inflammatory cells in X-irradiated animal on day 7 after infection. Direct immunofluorescence technique, using anti-mouse immunoglobulin G tagged with fluorescein isothiocyanate (×500).*

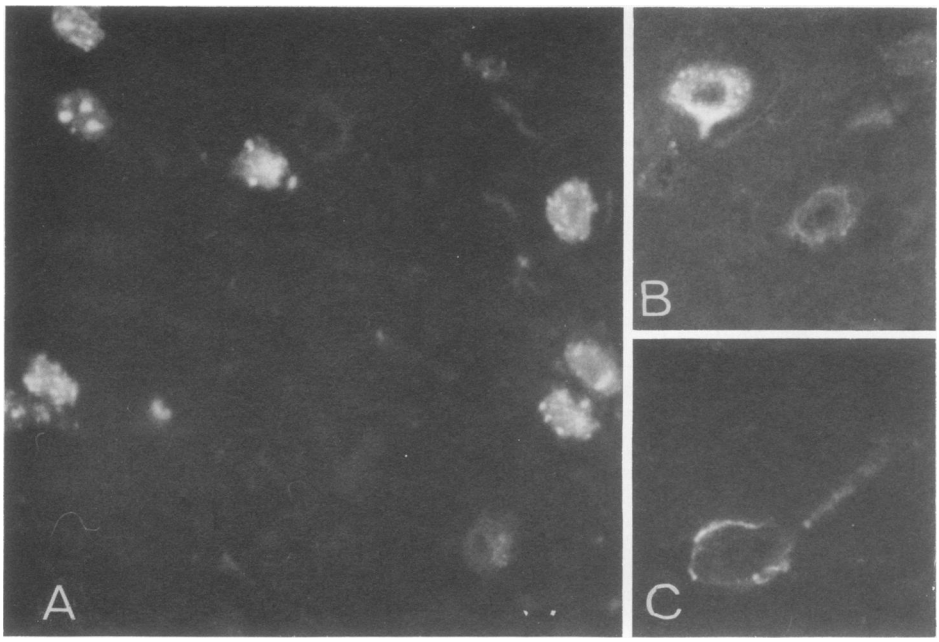



FIG. 6. *Vaccinia antigen in the cytoplasma of glial cells (A) and of a neuron (B), and on the cytoplasmic membrane in X-irradiated animals on day 18 after infection (C). (A) and (B) Fixed sections; (C) unfixed section (×500).*

TABLE 2. *Mice showing cytoplasmic immunofluorescence positive for vaccinia antigen in mesodermal cells and neuroectodermal glia and neurons*[a]

| Days post-infection | X-irradiated infected mice | | Nonirradiated infected mice | |
|---|---|---|---|---|
| | Mesodermal | Ectodermal | Mesodermal | Ectodermal |
| 0 | | | | |
| 4 | 2 (3) | 0 (3) | 3 (3) | 0 (3) |
| 7 | 5 (5) | 3 (5) | 2 (5) | 1 (5) |
| 18 | 3 (6) | 5 (6) | 0 (6) | 1 (6) |
| 60 | 0 (6) | 2 (6) | 0 (6) | 0 (6) |

[a] Number of mice positive; parentheses indicate number of mice examined.

capable of inducing two types of infection of the central nervous system. In immunologically competent animals, an acute, productive, one-shot (monophasic) infection develops which remains restricted to neural membranes, vessels, and inflammatory cells. Rapid virus replication is associated with the lysis of the host cell. Similar findings have been reported by others (3, 5).

The host reacts with a complex of defense mechanisms which clear the virus in about 8 to 10 days. As a rule, this type of infection is irrelevant and often remains clinically latent. In cases with extensive inflammation and cell damage, the permeability of the brain-barrier system increases pathologically and multiple lesions in adjacent neuroparenchyma become apparent, including damage of myelin sheaths.

The observation of similar lesions after injection of hypertonic saline into the carotid artery (J. Simon, unpublished data) suggests that such lesions are nonspecific, probably due to water, electrolyte, and acid-base imbalance. Other factors, e.g., mediators of inflammation (lysosomal enzymes, lymphokines, vasoactive amines), may also induce additional damage, designated as the "bystander effect" (17).

Most of such lesions are probably reversible, since they were rarely found by day 60 after infection.

Another type of infection, consisting of two phases, develops in some X-irradiated animals. The first phase begins with the above-mentioned productive, lytic infection of the neural membranes and vessels, with severe damage to the brain-barrier system. The second phase starts with the spread of infection into adjacent neuroparenchyma. A noncytocidal infection is established, which can persist during the whole observation period. Subsequently, inflammatory infiltrates appear in infected areas, and damage of the glial cell is seen. Myelin staining reveals perivascular, subpial, and subependymal demyelination.

The host reacts with further immune mechanisms. Our knowledge of these is only fragmentary. The methods used reveal antiviral antibodies, complement-binding antibodies toxic for em-

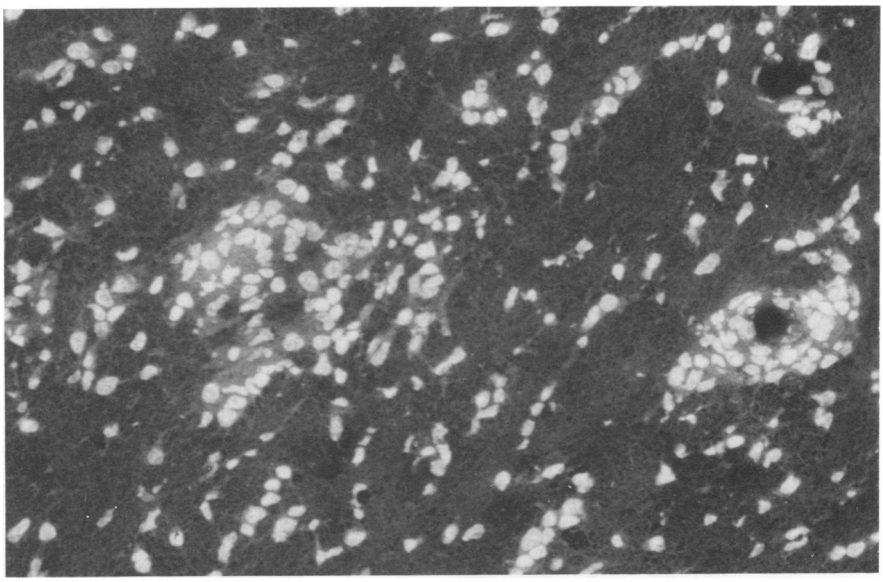


FIG. 7. *Perivascular and interstitial infiltrates in X-irradiated animal on day 18 after infection (acridine orange staining, (×500)).*

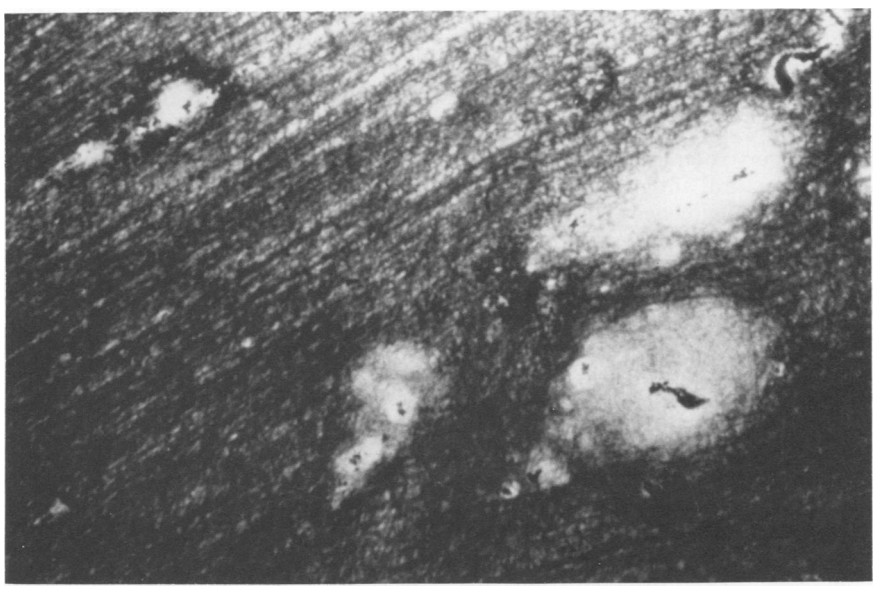


FIG. 8. *Perivascular demyelination in the white matter of X-irradiated animals on day 18 after infection (×500).*

bryonal glial cells or glioblastoma cells, and non-complement-binding antibodies with a specific affinity for glial cells and myelin sheaths. Unfortunately, there is no information on cellular immunity or the function of the immunological system. Lower titers of antiviral neutralizing antibodies in X-irradiated animals suggest some deficiency of the immune system.

The in vitro assay of antiglial toxic activity provides no information on the significance of such activity in vivo. The low activity detected in nonirradiated animals indicates that the in vitro system is highly sensitive. Surprisingly enough, similar differences have been found between multiple sclerosis patients and healthy persons (P. Mar, T. Gradl, and Ch. Dörner, J. Neurol. Sci., in press).

The pathogenesis of the first phase seems to be relatively simple. Direct, cytocidal virus action is the major determinant of the virus-cell

encounter. Efficient defense mechanisms of the host stop the process in most cases.

In contrast, the pathogenesis of the second phase, which may represent a rare complication of the first phase, is poorly understood at best. Deficient clearance of the virus may be one of the prerequisites for the second phase. Damage to the brain-barrier system associated with nonspecific lesions of adjacent nervous tissue may be another factor promoting the establishment of a noncytocidal infection in neuroectodermal cells. It is not clear what virus-host cell relationship may develop and what factors are involved. A crucial point seems to be the highly varying permissivity of cells of the central nervous system for vaccinia virus. Whereas the mesodermal cells of the neural membranes and vessels and some inflammatory cells are highly permissive for the strain used here, the neuroectodermal cells are nonpermissive or semipermissive. The only exception is the permissive ependymal cells. Preferential infection of the glial cells as a result of an increased permissivity in activated cells remains a speculation, based on the finding that other cells, e.g., lymphocytes, are permissive for some viruses only in activated form (8, 9).

The pathogenetic significance of the noncytocidal infection remains to be clarified. In vitro experiments show that vaccinia virus or its parts code for early antigens (surface antigens). These antigens appear very soon after infection before replication of structural antigens (1, 2, 16). The capability of demasking concealed host-specific antigens is also reported (15). However, it is not known whether membrane neoantigens are expressed in vivo. The immunofluorescence technique using unfixed brain sections supports this assumption, revealing membrane fluorescence of brain cells (indirect technique with antivaccinia serum). Confirmation of these findings with other methods is necessary. At present we can only speculate that the antigenic "makeup" of cytoplasmic membrane may be altered in noncytocidally infected cells. This may induce a complex of immune mechanisms, aimed at rejection of "not-self" cells. Consequently, the virus action would be indirect in this phase, mediated by immune mechanisms. The latter may act as a major effector of demyelination. Ongoing experiments employing other in vivo and in vitro systems and indicators of cell-mediated immune responsiveness should place our first results in context and provide an additional test of the immunological hypothesis previously proposed for postinfectious encephalomyelitis (6).

## ACKNOWLEDGMENTS

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