PATHOGENICITY OF A VACCINE STRAIN OF VACCINIA VIRUS IN RABBITS ADMINISTERED BY INTRAVENOUS ROUTE

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SUMMARY: The pathogenicity of the Lister (Elstree) strain, a vaccinia virus, in rabbits after intravenous injection was studied by histopathological and immunofluorescent methods. Inoculation of $1 \times 10^7$ PFU (pock-forming unit) virus into weanling and young adult rabbits caused severe emaciation and high mortality within 2 weeks. Pathological findings were characterized by vesicular lesions along mucocutaneous junction areas of the eyes, nose and mouth and by inflammatory changes in the brain, mainly in the meninges and choroid plexus. Immunofluorescent staining of the tissues of animals sacrificed at intervals demonstrated the accumulation of vaccinia viral antigen(s) in the loci of pathological changes. The suitability of this model system for the study of pathogenesis of human postvaccinal meningoencephalitis is discussed.

INTRODUCTION

Postvaccinal encephalitis (PVE) has been the most dreaded complication of the smallpox vaccination with high mortality and sequelae among survivors. The etiology and pathogenesis of PVE have been subjects of much controversy. Close correlation of PVE with major reaction of vaccination locus has been pointed out and viral pathogenicity as a trigger of an allergic mechanism was supposed to be responsible for the pathogenesis of PVE. Attempts to isolate infectious vaccinia virus from the brain tissue of PVE cases gave controversial results. Thurnbull and McIntosh (1926) and Angulo, Pimenta-de-Campos and de Salles-Comes (1964) reported isolation of vaccinia virus from the brain tissue of lethal PVE cases. Gurvich, Movsesyants and Stepenenkova (1975) isolated virus from cerebrospinal fluid from 5 non-lethal PVE cases. But there are much more reports of negative virus isolation from the central nervous system of PVE cases (Greenberg and Applebaum, 1948; Verlinde, 1951; Terzin et al., 1974; Kurata, Aoyama and Kitamura, 1977). Okudaira et al. (1973) and Kurata, Aoyama and Kitamura (1977) demonstrated the presence of vaccinia virus antigen in meninges and neurons in the brains of fatal PVE cases by immunofluorescence (IF) staining, with several cases giving positive IF staining only after treatment with NaSCN.
to dissociate the antigen-antibody complex (Dandliker et al., 1967). Virus isolation was unsuccessful in all cases (Kurata et al., 1977). These findings strongly suggested the participation of virus pathogenicity in the genesis of PVE syndrome.

Experimental studies of vaccinia pathogenicity have been attempted in mice (Cassel, 1957; Briody, 1959; Soekawa et al., 1974; Ginsberg and Johnson, 1976) by intracerebral or other peripheral inoculations, and in rabbits and monkeys (Hurst and Fairbrother, 1930; Rivers, Sprunt and Berry, 1933; Hashizume et al., 1973) by intracerebral inoculations. A comparative study of the neurovirulence of vaccinia virus strains has been done recently (Morita et al., 1977) and pathogenicity in the central nervous system was defined as a specific marker of each strain. The observation of the pathogenicity of vaccinia virus after peripheral or extraneural inoculation, however, has not been reported so far in detail.

The present study was carried out as a preliminary test for pathogenicity in rabbits of an usual vaccine strain of vaccinia virus administered by peripheral routes. The results suggest the ability of dermotropic vaccinia virus to reach the central nervous system. Possible suitability of this system as an animal model for the pathogenesis of PVE in human cases was suggested and discussed.

**MATERIALS AND METHODS**

*Rabbits*: Weanling (3-week old) and young adult (5-week old) male New Zealand white (NZW) rabbits were obtained from the Experimental Animal Center of the Institute of Medical Science. Their freedom from previous orthopox virus infections was confirmed by checking the neutralizing antibody (N.A.) (Kitamura and Shinjo, 1972).

*Virus*: Dermotropic vaccinia virus, strain Lister (Elstree), was grown by two passages through chick chorioallantoic membranes (CAM) after receiving from W.H.O. in a form of calf lymph smallpox vaccine. The virus in CAM homogenate was partially purified by two cycles of fluorocarbon treatments and final suspension in PBS (phosphate-buffered-saline, Dulbecco) had an infectivity titer of about $1 \times 10^8$ PFU (pock-forming units on CAM)/ml. Before the inoculation, the virus suspension was treated by sonication with a microtip of Branson B12 model for 20 sec.

*Fluorescent antibody against vaccinia virus*: Fluorescein isothiocyanate (FITC) conjugate of rabbit antiserum against Lister strain vaccinia virus was prepared as described before (Kurata et al., 1977).

*Immunofluorescence*: Direct staining of cryostat sections of removed tissue fragments with four staining units of FITC conjugate and fluorescence microscopy were carried out as described before (Aoyama et al., 1974).

*Histological examinations*: Parts of removed tissue fragments were examined histologically after fixation with formalin, paraffin sectioning and hematoxylin-eosin (H.E.) or Luxol-fast blue (LFB) staining. Staining of rabbit immunoglobulins was performed with commercially supplied fluorescent antibody
against rabbit IgM and IgG (Hyland Laboratories, U.S.A.).

Rabbit inoculation and examinations: In experiment 1, six weanling rabbits, 3 weeks of age and weighing about 400 g, were inoculated with 1.0 ml of a diluted virus suspension ($5 \times 10^6$ PFU/ml) through the auricular vein, to be observed clinically and sacrificed at intervals during the period 5 to 8 days after inoculation (p.i.). In Experiment 2, 12 young adults, 5 weeks of age and weighing 900 to 1,100 g, were divided into groups of six rabbits to be inoculated each with 1.0 ml of either $1 \times 10^7$ or $1 \times 10^5$ PFU/ml dose of the virus through the auricular vein. The animals of group 2-1 (inoculated with $1 \times 10^7$ PFU/ml) were observed and sacrificed at intervals up to 21 days p.i. Those of group 2-2 (inoculated with $1 \times 10^5$ PFU/ml) were observed and sacrificed 21 days p.i. Representative organs (brain, spinal cord, lung, liver, adrenal gland, kidneys, abdominal muscles, various lymphnodes, spleen and oral lips) were removed from the sacrificed animals and frozen in N-hexan immersed in a dryice—acetone mixture ($-70$ C) to be stored at temperatures below $-70$ C until examination.

RESULTS

Clinical Observations

After the intravenous inoculation of vaccinia virus, all weanling rabbits in Exp. 1 and young adults in Exp. 2, group 2-1, which received high virus doses ($1 \times 10^7$ PFU) developed rhinorrhoea, lacrimation, palpebral sebum, slaver and rough fur on day 3 p.i., resulting in general asthenia revealed by crouching and reduced appetite. These symptoms continued until sacrifice or death. Smear preparations were made from these mucosal secretions. All weanling rabbits were sacrificed between 5 and 8 days p.i. In group 2-1, the first rabbit died 8 days p.i. and other four animals were killed within 14 days p.i. when they appeared moribund. The sixth animal was killed on day 21 p.i. In the succumbed or moribund cases, no typical nervous symptom such as paralysis and convulsion was observed. In group 2-2 which received lower doses of virus ($1 \times 10^5$ PFU), nasal and conjunctival secretions were observed in only one rabbit during the period 3 to 8 days p.i.; no other clinical signs were observed in this animal later and in other animals throughout the observation period. All animals were killed 21 days p.i.

Macroscopic Findings

Gross examinations at autopsy revealed marked congestion in the meninges, partial edema in the parenchyma of the brain and increased stiffness of the lungs in all weanling and 3 of group 2-1 animals. On oral lips, partial reddening and induration were observed at muco-cutaneous junction areas. Foci of partial hemorrhages were seen in the abdominal muscle layer of one rabbit in group 2-1.
Histological Findings

Smears from oral, nasal and ocular secretions and small fragments of organs removed were subjected to histopathological examinations after H.E. staining. Smears collected 3 to 5 days p.i. contained few desquamated epithelial cells, many granulocytes and fluid debris (Fig. 1). In oral lips, there was a marked change composed of vesicle formation and partial destruction of the epidermal layer, turning into necrosis with infiltration of granulocytes and a few round cells, and the inflammatory foci of cell infiltration were observed in the subepidermal layer (Figs. 2a and 2b). These lesions were concentrated in the mucocutaneous junction areas. In the brain, the meninges were the main tissue of changes. The meninges of all six rabbits of Expt. 1 and 3 out of 6 of group 2-1 showed thickening with infiltration of polymorphonuclear leukocytes, lymphocytes and histiocytes and severe congestion or hemorrhage (Fig. 3). The ependymal layer of the ventricles was partially desquamated or disarranged with infiltration of granulocytes and lymphocytes. In the brain parenchyma, perivascular cuffing and small foci of infiltration by glia cells or lymphocytes, plasma cells and granulocytes were detected to be distributed diffusely in the submeningeal or periventricular areas (Figs. 4, 5 and 6). Degeneration or destruction of neurons was detectable but very few in number. Perivascular demyelination was not observed. Edematous loosening of brain substrate was also detected in some parts. Typical interstitial pneumonitis was found in animals of Expt. 1 and group 2-1. Adrenal cortex had marked infiltration of granulocytes and lymphocytes. In one case of group 2-1, Glisson's sheath of the liver showed infiltration of granulocytes and lymphocytes, accompanied by petechial hemorrhages along the abdominal wall.

Immunofluorescence Findings

Immunofluorescence studies were carried out on all organs of all rabbits to detect the distribution of vaccinia viral antigen(s). Results are summarized in Table I. In the nervous system, specific fluorescence was detected in the meninges (Fig. 7), choroid plexus and spinal cord, but not in the neurons. In other organs, specific staining was detected in the lung (Fig. 8), adrenal cortex (Fig. 9), muscle cells of abdominal wall (Fig. 10), epidermal layer of oral lips in wedge shape or vesicle formation (Fig. 11a and 11b), hair follicles and smears of oral lip, nasal and ocular secretions (Fig. 12). No viral antigen was detected in the kidneys or lymphnodes. Deposits of both IgM and IgG were observed in the meninges, choroid plexus and perivascular regions of parenchyma in fair agreements with the distribution of viral antigen.

Discussion

The present experiments were designed to examine the pathogenicity of vaccinia virus inoculated by an extraneural route (intravenous infection) into
Fig. 1. Smear of nasal secretion with desquamated and degenerated cells and granulocytes. Five days after infection (p.i.) (Group 2-1, Expt. 2). Hamatoxylin and eosin staining (H.E.) ×1000.

Fig. 2a. Destroyed epidermal layer with inflammatory cell infiltration and vesicle formation. Ten days p.i. (Group 2-1, Expt. 2). H.E. ×100.
Fig. 2b. Subepidermal layer with infiltration of granulocytes and lymphocytes. Ten days p.i. (Group 2-1, Expt. 2). H.E. ×250.

Fig. 3. Thickened meninges with infiltration of polymorphonuclear leukocytes, histiocytes and lymphocytes. Twelve days p.i. (Group 2-1, Expt. 2). H.E. ×250.
Fig. 4. Choroid plexus infiltrated by plasma cells, lymphocytes and granulocytes. Twelve days p.i. (Group 2-1, Expt. 2). H.E. ×250.

Fig. 5. Perivascular cuffing in the parenchyma of the parietal lobe (Group 2-1, Expt. 2). Ten days p.i. H.E. ×250.
Fig. 6. Glial node in the periventricular parenchyma. Ten days p.i. (Group 2-1, Expt. 2). H.E. ×250.

Fig. 7. Vaccinia viral antigen in the meninges stained by FITC-labeled antivaccinia antibody. Ten days p.i. (Group 2-1, Expt. 2). ×250.
Fig. 8. Specific fluorescence in the lung (Group 2-1, Expt. 2). Twelve days p.i. ×250.

Fig. 9. Specific fluorescence in the adrenal cortex. Ten days p.i. (Group 2-1, Expt. 2). ×250.
Fig. 10. Viral antigen in the muscle layer of abdominal wall. Eight days p.i. (Group 2-1, Expt. 2). ×400.

Fig. 11a. Specific fluorescence observed in the oral lip showing wedge shape. Twelve days p.i. (Group 2-1, Expt. 2). ×250.
Fig. 11b. Specific fluorescence found in the vesicular wall in the oral section. Twelve days p.i. (Group 2-1, Expt. 2). ×250.

Fig. 12. Viral antigen in the smear of oro-nasal secretion. Five days p.i. (Group 2-1, Expt. 2). ×400.
TABLE I

Distribution of vaccinia virus antigen revealed by immunofluorescence (IF) in weanling and young adult rabbits after intravenous inoculation of vaccinia virus

<table>
<thead>
<tr>
<th>Experiment Group</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Inoculum dose (PFU)</td>
<td>$5 \times 10^6$</td>
<td>$1 \times 10^7$</td>
</tr>
<tr>
<td>Organs tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>6/6</td>
<td>3/6</td>
</tr>
<tr>
<td>Meninges</td>
<td>6/6</td>
<td>3/6</td>
</tr>
<tr>
<td>Choroid plexus</td>
<td>NT</td>
<td>1/6</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>NT</td>
<td>4/6</td>
</tr>
<tr>
<td>Lung</td>
<td>NT</td>
<td>1/6</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>NT</td>
<td>0/6</td>
</tr>
<tr>
<td>Liver</td>
<td>NT</td>
<td>0/6</td>
</tr>
<tr>
<td>Abdominal muscle</td>
<td>NT</td>
<td>0/6</td>
</tr>
<tr>
<td>Kidney</td>
<td>NT</td>
<td>0/6</td>
</tr>
<tr>
<td>Lymph node</td>
<td>NT</td>
<td>0/6</td>
</tr>
<tr>
<td>Oral lip</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>Smears of oral lips, nasal and ocular secretion</td>
<td>6/6</td>
<td>6/6</td>
</tr>
</tbody>
</table>

* Groups of rabbits of designated ages were inoculated with designated doses of Lister (Elstree) strain of vaccinia virus via auricular vein and the organs were dissected at the death or moribund status (Expt. 1 and Group 2–1, Expt. 2) or at the termination of observation for 21 days (Group 2–2, Expt. 2).

*b 6/6: Number of animals with IF-positive specimens/number of animals tested.

c NT: Not tested.

weanling and young adult rabbits by histopathological and immunofluorescent (IF) methods, in special reference to invasion of the virus into the central nervous system (CNS). Reports on the pathogenicity of dermotropic vaccinia virus after extraneural inoculations have been relatively few and analyses were rather fragmentary. Intravenous injections of relatively high doses of Lister (Elstree) strain vaccinia virus, $5 \times 10^6$ PFU into weanling and $1 \times 10^7$ PFU into young adult rabbits, induced acute infection with severe emaciation and early, high mortality. Lower doses of virus ($1 \times 10^5$ PFU) did not produce any detectable symptom in young adult rabbits.

The most marked changes were found in the muco-cutaneous tissues, being characterized by exasperated secretion and partial desquamation of the epidermal layer. IF staining of mucos secretion smears and tissue sections demonstrated the accumulation of viral antigen(s), in correspondence with the loci of changes revealed by macroscopic observation and H.E. staining in rabbits at stages of clinical illness from onset to moribund status. Appearance of muco-cutaneous lesions in animals infected with vaccinia virus by a peripheral route has not been reported before. Vesicle formation and destruction of the epidermal layer
accompanied with inflammatory cell infiltration and the accumulation of viral antigen(s) along the muco-cutaneous junction areas (Figs. 2, 3 and 11) seem to suggest a specifically high sensitivity of this tissue to vaccinia virus infection.

In the central nervous system, the IF study of the brains revealed the appearance of vaccinia antigen in the meninges and choroid plexus, but not in the parenchyma which had many perivascular cuffing and foci of glial or inflammatory cell accumulation. Perivascular demyelination, the most characteristic feature of human PVE, has not been noticed in the rabbit brains in the present experiments. Verlinde (1951) failed to produce demyelination in animal experiments and suggested an indirect role of the vaccinia virus that produces inflammatory lesions in the brain but has no direct action on the myelin sheaths. In monkeys injected intracerebrally, the infectious virus can be recovered at high titers (Hashizume et al., 1972; Morita et al., 1977), whereas isolation of vaccinia virus from human PVE cases has been limited (Turnbull and McIntosh, 1926; Angulo et al., 1964; Gurvich et al., 1975). Despite the positive virus isolation from the diseased brain, Angulo et al. (1964) suggested rather a bigger role of the allergic mechanism from histopathological findings. IF studies by Okudaira et al. (1973) and Kurata et al. (1977) showed the presence of viral antigen(s) in the brain of lethal PVE cases. When these observations in human PVE cases and animal experiments of CNS inoculations are compiled, demonstration of vaccinia antigen by IF in the present experiments in the meninges and choroid plexus of rabbits after intravenous inoculation may be interpreted as suggesting that pathological changes characteristic of PVE should be correlated closely with the activity of infecting virus to cause the production or accumulation of viral antigen in CNS. Comparison of vaccinia virus strains for their capabilities of causing CNS findings after intravenous inoculation into weanling or young adult rabbits may give useful information about the suitability of this system as an animal model of human PVE and may serve for selection of safer vaccine strains to lower the incidence of PVE. Another experiment with the full check of the content of infectious virus in IF-positive tissues should be necessary to make thorough discussion on the pathogenicity or virulence of dermotropic vaccinia virus after intravenous inoculation.

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References


