ISOLATION OF A PARAVACCINIA VIRUS 
FROM A COW IN JAPAN

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(Received for Publication, March 1, 1967)

Poxviruses of paravaccinia subgroup which were proposed by Peters et al.13) have been isolated from cows in a variety of countries8,10~12,14). However, in Japan, very few incidence of pox disease of cows has been reported without isolation of the virus15,16). Most recently, however, the authors have succeeded in isolation of two strains of poxvirus, which were identical to vaccinia virus, from an outbreak of cowpox-like disease in 196215,16). After the outbreak, similar disease was reported in Hokkaido9) and in Saitama prefecture7), although in the latter cases attempts of isolation have failed.

A further investigation was continued to reveal that the presence of paravaccinia virus in bovine population may be a possible causal agent of the disease. This paper will be the first report of the isolation of a paravaccinia virus from a cow in Japan.

MATERIALS AND METHODS

Tissue culture

The testicles from new-born-cattle were trypsinized and monolayer cells were grown in Earle balanced salt solution containing 0.5% lactalbumin hydrolysate, 0.01% yeast extract, (ELY) and 10% bovine serum. The serum was reduced to 3% or no serum was used in maintenance medium.

Viruses

Isolate: The isolate, designated as PB-I is described in the text. A clone of the virus, which was made by 3 limiting dilutions in bovine testicle (BT) cells, was mainly tested for virus properties.

Vaccinia virus: Smallpox-vaccinia “Ikeda” which was described elsewhere16), was used as known control poxvirus.

Titration of the isolate

Serial 10-fold dilutions of the isolate were made in ELY and 0.1ml of each dilution was inoculated into cell sheet of BT. After 3 hours’ adsorption, each tube was added with 2ml of the maintenance medium described above. TCID₅₀ titer was calculated by Behrens-Kärber’s method9).

Serum-neutralization test

Anti-serum against smallpox-vaccinia “Ikeda” prepared in rabbits has been previously described9). Equal volumes of the serum diluted to one-tenth and the isolate PB-I at 100 TCID₅₀ were mixed and incubated at 37°C for 1 hour. The mixtures were then allowed to stand at 4~6°C for additional 21 hours. Thereafter, 0.1ml of the virus-serum

mixture was inoculated into cell sheet of BT. After 3 hours' adsorption, inoculated tubes were added with 2 ml of maintenance medium. Also mixtures of the anti-serum and 100 TCID$_{50}$ of smallpox-vaccinia “Ikeda”, and ELY and these viruses were tested by the same procedure.

Reactivation test
Reactivation test was done by the method described by Joklik et al. Smallpox-vaccinia “Ikeda” (5.64 log$_{10}$ pock forming units/ml) was used as reactivable virus after heating at 55°C for 120 minutes. One-tenth ml of mixture of the reactivable virus and the isolate PB-I (5.5 log$_{10}$ TCID$_{50}$/ml) was inoculated on the choiroallantoic membranes (CAM) of chicken embryonated eggs at 11 days old. On the 4th day after inoculation, pock lesion on the CAM was examined.

Morphological examination of the isolate
The supernatant fluid of the culture fluid of BT cells infected with the isolate PB-I, derived by centrifugation at 2500 r.p.m. for 10 minutes, was employed as starting material. A part of the virus containing supernatant fluid was serially passed through Millipore filter in order to estimate the approximate size of the virus particles. The other part of the fluid was purified by repeating two cycles of centrifugation (2500 r.p.m. for 10 minutes and 32000 g for 20 minutes). The sediment was finally suspended in distilled water. Droplets of the resulting suspension were negatively stained with sodium phosphotungstate on carbon-coated copper grid. The specimens were examined by JEM-7 electron microscope (Japan Electron Optics Laboratory Co., LTD., Tokyo, Japan).

RESULTS

Isolation of the virus
According to the owner of a farm in Chiba prefecture, pox-like lesions were recognized in June, 1966, in 7 of 9 Holstein-Friesian cows kept in his farm. The cow initially affected had been purchased and brought into the farm in the middle of May from Fukushima prefecture. When all the diseased cows were healed about the middle of June, a Holstein-Friesian cow of 5 years old was brought into the farm from Yamagata prefecture. Within a week the owner found one reddish pustule on the hind-left teat of the cow. After a few days, many pustules, as about the size of red or soya beens appeared on the 3 teats and the udder. When one of the authors (G.M.) examined the cow early in July, many pustules had been already ruptured and followed by dark reddish scabs (Fig. 1). Pain was not noticeable and the scabs were easily removable from the skin. Scars were not observed under the scabs. One teat whose skin was intact was found suffering with mastitis.

The scabs and pustules from the affected cow, were harvested and disintegrated in glass homogenizer with ELY containing 2000 units penicillin and 1000 µg streptomycin per ml. The suspension was kept at 4~6°C for 18 hours and then centrifuged at 2500 r.p.m. for 10 minutes. The supernatant fluid was mixed with a trypsinized suspension of BT cells at the ratio of 1:19 and the mixture was seeded into tubes and incubated at 37°C. After 21 days, a few foci (Fig. 2) were observed. The cells in the foci were swollen, rounded and granular. Sometimes cells were refractile. This cytopathic effect (CPE) was followed by the detachment of degenerated cells from the glass walls. Within 4 days, this CPE extended almost throughout the cell sheet. For next passage, the culture was freeze-thawed and disintegrated in glass homogenizer. After a light centrifugation, one volume of the supernatant fluid was mixed with 9 volumes of the BT cell suspension and 1ml each of the mixture was distributed into each tube. On the
4th day, similar CPE was observed.

The cytopathic agent has been carried for 10 consecutive passages in BT culture. Titers of infected culture fluid were 4.5–5.5 log_{10} TCID_{50} per ml.

For cytological examination of the CPE, cell cultures grown on cover-slips were fixed and stained by hematoxylin and eosin or Giemsa. At the early stage of the infection a few eosinophilic granular inclusion bodies appeared in cytoplasm. Then they became larger and usually surrounded nucleus (Fig. 3). At that stage, cytoplasm showed numerous vacuolation in its periphery.

Serum-neutralization test
Anti-vaccinia serum did not neutralize the isolate PB-I.

Reactivation test
As shown in Table 1, no pock was demonstrated on the CAM of the chicken embryonated eggs which was inoculated either with the isolate PB-I or with heat-inactivated vaccinia virus, and no active virus was detectable at least on the 2nd passage. The CAM inoculated with the mixture of reactivable virus and the isolate PB-I revealed pocks which were evidenced by neutralization test to be induced by vaccinia virus.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Direct pock count</th>
<th>Sub-inoculation of ground membrane</th>
<th>Neutralization with anti-vaccinia serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-vac* + PB-I 10^0</td>
<td>3**, 4, 2, 1</td>
<td>positive</td>
<td>neutralized</td>
</tr>
<tr>
<td>H-vac + PB-I 10^{-1}</td>
<td>0, 0, 0, 0</td>
<td>positive</td>
<td>neutralized</td>
</tr>
<tr>
<td>H-vac + ELY</td>
<td>0, 0, 0, 0</td>
<td>negative</td>
<td>***</td>
</tr>
<tr>
<td>ELY + PB-I 10^0</td>
<td>0, 0, 0, 0</td>
<td>negative</td>
<td>****</td>
</tr>
</tbody>
</table>

* Heat-inactivated vaccinia virus 'Ikeda'.
** Number of pock on each CAM.
*** Not tested.

Morphological examination of the isolate
The isolate passed through Millipore filter discs having an average pore diameter of 450, 300 and 220 m\mu but did not pass through disc having an average pore diameter of 100 m\mu.

By the electron-microscopy, virus particle of the isolate was observed to have the characteristic tubular thread structure on the surface of the particles and crisscross or woven pattern with the thread (Fig. 4). The virus particles were measured to be 129–133×243–287 m\mu in diameter and the thread on the surface of the particles was 80–100 Å in wide.

DISCUSSION

The viral isolate, designated as PB-I, from pox-like lesions of a cow was capable to reactivate vaccinia virus and was not neutralized by anti-vaccinia serum. The structure of the virus particles was shown to resemble that of the known paravaccinia viruses. On the basis of the above findings the isolate was considered to belong to the paravaccinia subgroup which includes viruses of contagious pustular dermatitis (CPD) or orf, bovine papular stomatitis and milker's nodules (pseudo-cowpox)\textsuperscript{18}.

Very few study on paravaccinia viruses has been presented in Japan. \textit{Asakawa et al.}\textsuperscript{2} reported contagious erythema (CPD)-like disease occurred in 1948 in sheep kept in
National Institute of Health, Tokyo, Japan, and a outbreak of the same disease in 1950 in Yamanashi prefecture. They could not isolate viruses by inoculating the specimen to the CAM, cell culture or laboratory animals. Ishii et al. reported the isolation of CPD virus from sheep and goat in Nagano prefecture in 1952. The size of the virus particle was 200×250 nm in diameter.

Classification of paravaccinia viruses is based on animal species from which the virus was isolated, rather than on virological characters of the viruses. Nairnington et al. reported that milker's nodule virus was morphologically identical to orf virus. According to Huck, a virus which was isolated from a cow's teats lesion was neutralized by orf anti-serum in tissue culture and experimentally caused lesion on cow's teats but did not cause a lesion on sheep skin. If the animal species from which the virus was isolated, was considered of prime importance, our isolate would be termed pseudo-cowpox virus.

**SUMMARY**

A paravaccinia virus was isolated on cell culture from pox-like lesions on the teats of a cow.

**ACKNOWLEDGMENTS**

The authors express their gratitude to Prof. R. Yanagawa and his associates of Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan, for electron microscopic examination and to Dr. H. Ito of the Kastuho Branch, the Chiba Prefectural Federation of Agricultural Insurance Associations for his assistance of field surveys of the disease.

**REFERENCES**

近来、諸外国においてバラクチニア・サブループに属するボックス・ウイルスが牛から分離報告されているが、全国においては未だその例を見ない。

本報告は、昭和41年夏、千葉県において牛からバラクチニア・サブループに属すると思われる1株のウイルスを分離したので、その分離状況、ならびに同定成績について記載するものである。

ウイルスの分離

千葉県の1農場において導入して間もない1頭の牛の乳頭および乳房に多数の赤色の発症を認めた。一つの乳頭を摘出したところ、21日目に細胞変性を認め、この細胞変性は細胞の円形化とフォーカス形成を主徴とし、主に細胞シート全域に拡がった。

また感染細胞原形質内には好酸性の著入体を認めた。

ウイルスの同定

分離ウイルスは発育部位の膿皮膜上にボックスを形成せず、またウイルス・ウイルスの免疫血清中和されなかったが、熱不活化したウイルス・ウイルスを再活性化した。本ウイルスは平均孔径220 mμのMillipore filterを通過したが、100 mμのものは通過しなかった。ネガティブ染色試料の電子顕微鏡観察により、ウイルス粒子の大きさは129～133 ×243～287 mμと推定され、またウイルスの構造はバラクチニア・サブループに属する既知ウイルスの記載と極めて良く一致した。
以上のご成績から今回の分離ウイルスをバラクチニア・ウイルスと同定した。