STUDIES ON THE COWPOX-LIKE DISEASE OUTBREAK IN HOKKAIDO, JAPAN

II. PROPERTIES AND IDENTIFICATION OF THE ISOLATES

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INTRODUCTION

An outbreak of cowpox-like disease occurred in Hokkaido, Japan, in the fall of 1962. From the skin lesions of cows exposed to the outbreak, viral agents which were similar to animal pox viruses were isolated as previously reported⁴¹⁾.

This paper deals with properties of the isolates as compared to vaccinia and cowpox viruses. The identification of the isolates and naming of this disease in cattle are discussed.

EXPERIMENTAL MATERIALS AND METHODS

Viruses

Viruses employed in this experiment were those isolated as the causative agents of cowpox-like disease, and were pox viruses used as the test control.

Isolates: Two strains of isolates were mainly tested for their properties. As previously described, these strains were isolated from skin lesions of cows on the chorioallantoic membrane (CAM) of chicken embryonating eggs and HeLa cells. For the isolation, serial passages through cow skin (2 successive passages), rabbit skin and testicles were employed. One of the two strains of isolates was labelled as II and the other as VII₃, in order to provide an identification number of the skin lesions of cows from which they originated.

Pox Viruses: Pox viruses used as known control were as follows: cowpox "Umeno", cowpox "Downie" and smallpox-vaccinia "Ikeda".

Cowpox "Umeno": A strain of cowpox virus which was isolated in Inch'on, Korea by Shinkichi UMENO⁴²⁾ in 1906 from a cow with a spontaneous cowpox. Since its isolation, the strain has been maintained in this laboratory by employing serial passage only on cattle skin. In this experiment the virus was used in the dermal pulp of infected cows, culture fluid of infected HeLa cells, and suspension of infected CAM of chicken embryonating egg.

Cowpox "Downie": A strain of cowpox virus, isolated by A. W. DOWNIE, was provided by Isamu TAGAYA of the National Institute of Health, Japan. This strain was lyophilized after its infection on the CAM of chicken embryonating egg. The ampule was labelled as "LB red E 38". The state of the virus used in this experiment was emulsion of the infected CAM and culture fluid of HeLa cells infected with the virus.

Smallpox-vaccinia "Ikeda": This strain of vaccinia virus derives its origin from the skin lesion of a woman affected with smallpox during its occurrence in the summer of 1929

in Osaka City, Japan. Takeo IKEDA successfully transmitted the lesion to a cow through rabbit testicle and he vaccinified this virus. A commercial vaccine against the smallpox is prepared in this Institute from the dermal pulp of cows artificially infected with this smallpox-vaccinia virus. The virus employed in this experiment was a dermal pulp of an infected cow, emulsion of an infected CAM, and a culture fluid of infected HeLa cells.

For cloning of all the viruses, isolates and pox viruses, limiting dilution was repeated five times in HeLa cell culture.

Cell Cultures

A line of HeLa cell designated as "S3" and monolayer of primary cells, derived from the kidneys and testicles of various animals, were prepared as previously described⁴¹⁾.

Preparation of Antiserum

Convalescent and immune sera against isolates and control pox viruses were prepared in rabbit as shown in Table 1. These antisera were used in the preliminary tests of cross serum-neutralization and cross complement-fixation.

Table 1. Method of Preparing Convalescent and Immune Rabbit Sera against Isolates II and VII₃, and Control Pox Viruses

The Preliminary Inoculation* ¹			The Secondary Inoculation			
Inocula	Source of Inocula	Result	Days* ⁶	Inocula	Result	Bleeding off (days* ⁶)
II	material labelled as II* ² →Cs* ³ →Cs→Rs* ⁴ →Rt* ⁵ →CAM→CAM	take +++	•	not done	•	23
VII ₃	material labelled as VII ₃ * ² →Cs→Cs→Rs→Rt→CAM→CAM	take +++	•	not done	•	21
Smallpox-vaccinia "Ikeda"	dermal pulp of infected cow	take +++	16	The same as used with the preliminary inoculation	take —	22
Cowpox "Umeno"	dermal pulp of infected cow	take +++	16	The same as used with the preliminary inoculation	take —	29
Cowpox "Downie"	infected CAM	take +++	•	not done	•	21

*¹ Inoculation was conducted by dermal scarification.

*² Skin lesion obtained from a cow naturally infected with the disease.

The lesion was serially passed as shown by the mark, →.

*³ Cs=The skin of experimental cow.

*⁴ Rs=Rabbit skin which initially showed pustular lesions during the serial passage.

*⁵ Rt=Rabbit testicle which initially showed swelling and haemorrhages during the serial passage.

*⁶ Days after the preliminary inoculation.

Hyperimmunized rabbit sera against the isolates and the control pox viruses were prepared by multiple intraperitoneal inoculations of these viruses into rabbits which recovered from intradermal or subcutaneous exposure to the viruses. Intraperitoneal inoculation was repeated 3~5 times at 4-day intervals, starting about 2 weeks after the preliminary inoculation. Rabbits were bled 10~14 days after the final inoculation. The virus materials used as to inoculate to these rabbits were culture fluid of HeLa cells infected with cloned viruses.

Morphology of Virus Particles of Isolate

The culture fluid of HeLa cells infected with isolate II, which was cloned by limiting dilution on HeLa cells, was examined for the morphology of virus particles. For the test, one part of the supernatant fluid of the culture fluid, derived by centrifugation at 1,000 r.p.m. for 10 minutes, was serially made through Millipore Filter. The other part of the

supernatant fluid was treated with fluorocarbon and was examined by electron microscopy. For the treatment, fluorocarbon was added to an equal volume of the virus-containing culture fluid and then homogenized at 20,000 r.p.m. for 75 seconds. The mixture was centrifuged at 1,000 r.p.m. for one minute. The supernatant fluid was recentrifuged at 10,500 G for an hour. Then, the sediment was resuspended in phosphate buffered saline and concentrated to 1/10 the volume of the original fluid. Droplets of the resulting suspension were air dried on a formvar-coated specimen grid, rinsed with distilled water, and then were shadowed with chromium. Specimens employed for negative staining were prepared by the method described by MAYOR et al³⁰⁾. In order to examine the structure of virus particles in a thin sectioned material, small blocks were excised from infected tissues, which had been fixed in 10% formalin and stored for routine histological examination. After a thorough washing with water, the tissue blocks were refixed at 0°C for 2 hours in 1% osmium tetroxide buffered at pH 7.4. They were dehydrated in graded dilutions of ethyl alcohol, embedded in methacrylate and thin sectioned. The specimens were examined by a JEM-type 6 S electron microscope.

Cross Protection Test

Experimental cows which were previously inoculated dermally with cow skin lesions of the original and experimentally produced and with smallpox-vaccinia were challenged dermally with the isolates and control pox viruses. The following cows were employed in the cross protection tests.

Experimental Cows: Experimental cows, Nos. 1, 2 and 3, employed in the test, were the same as those used in the isolation experiment of the causative agent of the disease. Details of these cows were previously described⁴¹⁾.

Experimental cow No. 4, Born & bred: Miyagi prefecture; Breed: Holstein-Friesian; Age: 4 years; Sex: Female; Body weight: 345 kg. This cow was inoculated with smallpox-vaccinia "Ikeda" in the preliminary inoculation. A 20% emulsion of the vaccinal dermal pulp of cow was rubbed into a cross-cut square of 4×10 cm made on the skin at the abdomen. Typical lesions and febrile reaction were observed in the cow. The lesions developed into a dry scab of black color with cracks when the cow was challenged for the protection test 15 days after the preliminary inoculation.

Experimental cow No. 5, Born & bred: Fukushima prefecture; Breed: Holstein-Friesian; Age: 4 years; Sex: Female; Body weight: 407 kg. This cow was used as a challenge control for the test. The preliminary inoculation was not conducted upon the cow.

Challenge: Challenge on the experimental cows, which were previously infected, was conducted 15~33 days after the preliminary inoculation. The inocula for the challenge were emulsions of the following materials: The skin lesions of cows labelled as II, III, VII₃ and XVI which were serially passed 2 times through the skin of experimental cows; Dermal pulp of cows infected with smallpox-vaccinia "Ikeda" and with cowpox "Umeno", and CAM infected with cowpox "Downie". The materials were emulsified to 2 and 10% concentrations in physiological saline containing penicillin, 1,000 units, and streptomycin, 500 µg per ml.

Prior to inoculation of the materials, cows were laid on their back to shave their flank. The site of inoculation was then surgically cleansed. Parallel cross-cuttings of 2 mm-interval in a square of 4×10 cm were made on the cleansed site. Inocula were rubbed into the scarified skin with the aid of an index finger which was covered with a sterile rubber sack.

A droplet of physiological saline containing penicillin and streptomycin and a suspension of *Staphylococcus albus*, isolated from the original skin lesion, were rubbed into the scarified area. This process was initiated in order to check the possibility of accidental vaccinal contamination during the experimental stage, and to examine the virulence of the staphylococcal isolate to the scarified cattle skin.

Serum-Neutralization Test

Serially diluted serum, which was inactivated at 56°C for 30 minutes, was mixed with an equal volume of 100~1,000 TCID₅₀ virus. The virus materials were the supernatant fluid, derived by centrifugation at 2,000 r.p.m. for 20 minutes, of culture fluid of HeLa cells infected with cloned viruses. The virus-serum mixture was incubated for an hour in a 37°C water bath and followed by storing at 4~6°C for additional 20~22 hours. A fifty per cent neutralizing end point of the test serum was calculated by the Reed and Muench method 2 weeks after the inoculation of this mixture into HeLa cells.

Complement-Fixation Test

Complement-fixation test was performed by the method described by PLOTZ et al³⁶⁾. Sera were inactivated at 56°C for 30 minutes. Two full units of guinea-pig complement were used. Antigens used were the supernatant fluid of a 25% emulsion, CAM of chicken embryonating eggs infected with isolates and control pox viruses. These emulsion of infected CAM were held at 4°C for one week, prior to obtaining the supernatant fluid. Two units of the antigens were used.

Hemagglutination-Inhibition Test

The hemagglutination-inhibition test conducted in this experiment was the method described by KITAOKA et al²⁸⁾. The virus-containing culture fluid of HeLa cells, which was used in the serum-neutralization test, were employed as hemagglutinin. Four units of the hemagglutinin were added to the sera previously inactivated at 56°C for 30 minutes, and the mixtures were incubated at 38°C. After an hour of incubation the result of the test was read.

Gel Diffusion Test

Gel diffusion test, using tubes, was the method described by GISPEN¹⁷⁾ for the analysis of pox virus antigens.

Antisera employed in the test were hyperimmunized rabbit sera used in the serological tests described above. Prior to the test, the sera were inactivated at 56°C for 30 minutes and employed in the test without dilution.

Antigens of the isolates and control pox viruses were prepared from infected CAM of chicken embryonating egg. The membranes were harvested 3 days after infection, and pooled batches of the membranes were minced and suspended in phosphate buffered saline; 0.5 ml of saline was added to 1 g of the membrane. The suspensions were held at 4°C for 3 days with 5 minutes shaking 3 times a day, and they were centrifuged for 15 minutes at 2,000 r.p.m. to remove the membrane debris. The centrifuged extracts were stored at -20°C and used as antigens.

EXPERIMENTAL RESULTS

BIOPHYSICAL AND BIOCHEMICAL PROPERTIES OF THE ISOLATES

Morphology of Virus Particle of Isolate

Serial filtration, through Millipore Filter (MF), of the culture fluid of HeLa cells which was infected with cloned isolate II resulted as shown in Fig. 1. The isolate contained in the fluid passed through MF discs having an average pore diameter (APD) of 800, 650, 450 and 300 m μ , but it did not pass through disc having an APD of 100 m μ . For a control test, smallpox-vaccinia "Ikeda", which was cloned and propagated in HeLa cells, was made through MF with an APD of 300 and 100 m μ . The virus was detected in the filtrate which passed through a disc with an APD of 300 m μ but not that of 100 m μ .

Virus particles of isolate II which were present in the culture fluid of HeLa cells, appeared as brick-like form with central upheaval upon electron microscope examination.

They were $240\sim 265 \times 295\sim 315$ m μ in diameter. As can be seen in Photo 1, the envelope membrane and cord structure subdivided into small tubular units were shown in the particle negatively stained with phosphotungstate. These findings were presumed to be an evidence that isolate II belonged morphologically to a group of pox viruses.

Fig. 1. Serial Filtration of Isolate II through Millipore Filter

cornea. Typical opacity of the cornea was observed after infection with infected CAM, which was obtained by 3 additional passages on the membrane of the lesional CAM described above.

Sensitivity to Ether

Ethyl ether was added to a concentration of 20% by volume to the supernatant fluid of the virus-containing culture fluid of HeLa cells. Tubes containing the mixture were capped with silicon-treated stoppers and shaken, and they were then maintained at 4°C for 20 hours. During the refrigeration period, the mixtures were shaken 3 times. This cold storage was followed by standing the mixtures at 37°C for an hour, after replacing the silicon stoppers with aluminium foil caps, in order to evaporate the ether. The virus-containing culture fluid, which were added with Hanks B. S. S. instead of ethyl ether, were used as non-treated controls.

Results of the test, which are summarized in Table 2, indicated that the sensitivity of isolates II and VII₃ to ethyl ether was similar to that of vaccinia and cowpox viruses, which are classed as a group of ether-resistant viruses.

Heat Stability

The supernatant fluid of the virus-containing culture fluid of HeLa cells was heated to 56°C in a water bath. After a 20-minute heat-exposure of the isolates II and VII₃, which

Table 2. Sensitivity of Isolates and Control Pox Viruses to Ethyl Ether

Viruses	Treated with	Virus Titer after Treatment (log TCID ₅₀ /ml)	Decrease in the Virus Titer after Treatment
Isolate II	ethyl ether	4.56	1.11
	Hanks B. S. S.	5.65	
Isolate VII ₃	ethyl ether	3.24	0.91
	Hanks B. S. S.	4.15	
Smallpox-vaccinia "Ikeda"	ethyl ether	4.48	1.12
	Hanks B. S. S.	5.60	
Cowpox "Downie"	ethyl ether	3.94	0.62
	Hanks B. S. S.	4.56	

respectively showed initial TCID₅₀ titers of log 6.07 and 6.34, a decrease in the titers to log 2.0 and 2.11, respectively, was noted. These decreases in titers indicated more than 99.9% reduction in infectivity of the isolates. Similarly, the virus titers of log 5.95, 6.39 and 5.7, which were respectively observed on pre-heating smallpox-vaccinia "Ikeda", cowpox "Umeno" and "Downie", dropped to log 2.3, 2.4 and 1.7, respectively after heating for 20 minutes. The deduction rate of the infectivity of these viruses was approximately the same as that observed with the two strains of isolate.

PATHOGENICITY OF THE ISOLATES

Pathogenicity for Mouse

Weaned dd mouse of female, 2 weeks of age and weighing 8 g, was exposed to the isolates and control pox viruses. Each mouse was inoculated intraperitoneally with 0.1 ml of the supernatant fluid, derived by centrifugation at 1,500 r.p.m. for 5 minutes, of a 10% suspension in Hanks B. S. S., of pock lesions on CAM produced with the viruses. The pocks on CAM of chicken embryonating eggs were harvested 3 days after chorioallantoic inoculation with the viruses. Ten mice were inoculated with each of the virus suspensions.

All the 10 mice inoculated with cowpox "Downie" were dead with the incubation period from 5 to 13 days. An average of the incubation period was 7 days. In autopsy findings upon the mice, heavy bleeding in the peritoneal cavity was the most remarkable. A large quantity of reddish and slimy liquid exuded from the peritoneal cavity, when the incision was made on the peritoneum. The cavity was markedly swollen by the presence of the liquid. Large haemorrhages were also observed in the ovary throughout the oviduct.

On the other hand, the mice inoculated with the isolates, smallpox-vaccinia "Ikeda" and cowpox "Umeno" survived without any of clinical abnormality.

Pathogenicity for Chicken Embryonating Egg

Histopathological examinations of infected CAM of chicken embryonating egg were performed with the following three specimens which were obtained through serial passage as indicated below.

Specimen No.	Source
1	original cow skin lesion II→Cs→Cs→Rs→Rt→CAM
2	original cow skin lesion VII ₃ →Cs→Cs→Rt→CAM
3	original cow skin lesion VII ₃ →Cs→Cs→Rs→Rt→CAM

Note: →indicates one passage. Cs=cow skin, Rs=rabbit skin, Rt=rabbit testicles.

Common finding observed in the ectoderm of the three specimens of membranes was an extensive proliferation of the cells. The ectoderm showed marked thickening with the presence of granular leucocytes and an occasional cellular necrosis. Haemorrhages were not observed. Ballooning of the ectodermal cells was found in specimen No. 2 in a high ratio. In specimens Nos. 1 and 2, inclusion-like bodies were observed in the cytoplasm of cells of the deep layers of the ectoderm. They were eosinophilic when stained with H. E. after formol fixative. The size varied from pin-point granules to that of the cell nuclei. The mesoderm showed slight oedema and congestion but not haemorrhage. Infiltration of large and round cells, histiocytes or the cells from RES was frequently observed. The infiltration of these cells in the mesoderm usually occurred around the congested vessels and in the area located adjacent to the thickened ectoderm. Some of these cells were coarsely swollen and vacuolated. The entoderm showed the presence of a few cells with slight proliferation and ballooning.

The noted changes described above were materially same as those reported by DOWNIE¹⁰⁾ in his study on lesions produced by cowpox virus on CAM. According to this report, numerous eosinophilic inclusion bodies were produced 48~72 hours after the infection with

this virus. However, in the present authors' specimens no more than a few inclusions or inclusion-like bodies were found.

Pathogenicity for Cell Cultures

The isolation of the causative agent of cowpox-like disease on cell cultures was previously reported⁴¹⁾. Cytopathogenic effect (CPE) of the isolates was characterized by rounding of the infected cells and the formation of infectious foci which developed into plaques.

In the early stage of the infection of cell cultures with isolates II and VII₃, the cells which were regarded to be infected with these isolates became round showing a slight expansion of the cells. Degeneration extended to the adjoining cells from those initially infected. These degenerated cells appeared as foci of whitish point on the cell sheet and which were clearly visible. Later, the cells involved in the foci showed lysis and then detachment from the wall of the culture vessels. In this stage the foci developed into plaques 1~2 mm in diameter. Generally, complete destruction of cell sheet was observed only when large amounts of the isolates were inoculated into the cell sheet. A majority of the sheet infected with small amount of the isolates usually remained normal, except for a certain number of the foci and plaques.

The CPE of the isolates was commonly observed upon HeLa cell, the primary monolayer cells of bovine embryonating kidneys, rabbit testicles and kidneys, and goat kidneys. However, the plaques formed by the isolates in HeLa cells were clearer than those observed in the other monolayer cells.

The CPE observed with the control pox viruses in these cell cultures was materially the same. However, the occurrence of plaque formation was delayed, and a slight weakness in cell lysis were occasionally observed only with cowpox "Downie"; slightly different from the isolates, smallpox-vaccinia "Ikeda" and cowpox "Umeno".

Cytoplasmic inclusion bodies were observed in the cell cultures which were infected with the isolates as shown in Photo 4. These inclusion bodies were of the matrix type. They were stained reddish purple with Giemsa after fixation with methanol. On the other hand, H. E. staining after Bouin fixation resulted in bright red inclusion bodies with clear halo. However, staining reaction of the inclusion bodies was very weak when they were fixed with formalin. In the early stage of the infection, the inclusion bodies appeared tiny and round which eventually increased in size and became irregular in shape. Finally, they diffused throughout the cytoplasm of infected cells. This type of inclusion bodies was commonly observed in the cell cultures infected with the control pox viruses as well as the isolates. This inclusion body, which can be reckoned as Guarnieri body, was similar to the "B" type inclusion as termed by SATO³⁹⁾, and KATO et al.²⁵⁾ who classified the cytoplasmic inclusion bodies due to infection of pox viruses into "A" and "B" types.

In addition to this type of inclusion body, another type of cytoplasmic inclusion body was observed only with cowpox "Downie" (Photo 5). These inclusion bodies were round, at all stages of the infection. They were homogeneous and of the hyaline type. When the material was stained with Giemsa after methanol fixation, they were stained blue or purple-blue, and when they were stained with H. E. after Bouin fixation they appeared red. These inclusion bodies can be the same as those described by DOWNIE¹⁰⁾, and they were similar to the "A" type inclusion termed by KATO et al.²⁵⁾ According to the investigators, the "A" type inclusions are significant marker in cowpox virus infections. They believe that animal pox viruses, other than cowpox, do not form this type of inclusion body in the cytoplasm of infected cells. It is interesting to note that this type of pox virus inclusion body was observed in the cell cultures only after the infection with cowpox "Downie" but not with the isolates II and VII₃, and the other control pox viruses, smallpox-vaccinia "Ikeda" and cowpox "Umeno".

Inclusion bodies observed in smear preparations, which were stained with Giemsa after

methanol fixation, of the cornea of a rabbit infected with isolate II were those of Guarnieri type as shown in Photo 6. These smear preparations were prepared from the rabbit 3 days after inoculation. At least, in these preparations, the hyaline type inclusion bodies were not observed. Photo 3 shows virus particles as revealed by electron microscope on thin section of this infected rabbit cornea.

IMMUNOGENICITY OF THE ISOLATES

Cross Protection Test

Table 3 shows the result obtained in cross protection test on experimental cows. These

Table 3. Result of Cross Protection Test on the Skin of Experimental Cows

Exp. Cow No.	The Preliminary Inoculation		Challenge after the Preliminary Inoculation									
	Inocula	Result (take ^{*4})	Days ^{*1}	Inocula								Saline ^{*3}
				II	III	VII ₃	XVI	Smallpox-vaccinia	Cowpox "Umeno"	Cowpox "Downie"	Staph. ^{*2} alb.	
1	The original skin lesions	±	33	—	—	—	±	+++	++	±	—	—
2	The original skin lesions	+	33	—	—	—	—	+	—	—	—	—
3	Skin lesions at the 2nd pass-level	++	28	—	—	—	—	++	++	—	—	—
4	Smallpox-vaccinia "Ikeda"	+++ (febrile reaction)	14	—	—	—	—	—	±	—	•	•
5	None	•	•	++	•	++	•	•	•	+++	•	•

*1 Days after the preliminary inoculation.

*2 Suspension of *Staph. albus* (10 mg/ml) isolated from the original skin lesion labelled as II.

*3 Physiological saline containing penicillin, 1,000 units, and streptomycin, 500 µg/ml.

*4 — : negative, ± : equivocal reaction, + : weak take, ++ : moderate take, +++ : strong take.

Table 4. The Antibody Response of Experimental Cows Employed in Cross Protection Test

Antibodies against			Experimental Cow Number			
			1	2	3	4
Antibody Titers before the Preliminary Inoculation	SN	II ^{*2}	< undil.	50	< undil.	not tested
		VII ₃	< undil.	124	< undil.	
		I	< undil.	124	< undil.	
		U	< undil.	60	< undil.	
		D	< undil.	70	< undil.	
	CF	II	< 8	< 8	< 8	< 8
		VII ₃	< 8	< 8	< 8	< 8
		I	< 8	< 8	< 8	< 8
	HI	VII ₃	< 8	8	< 8	< 8
		I	< 8	8	8	16
Antibody Titers after the Preliminary Inoculation ^{*1}	SN	II	< undil.	150	< undil.	not tested
		VII ₃	< undil.	110	< undil.	
		I	< undil.	60	< undil.	
		U	< undil.	50	< undil.	
		D	< undil.	60	< undil.	
	CF	II	< 8	< 8	< 8	< 8
		VII ₃	< 8	< 8	< 8	< 8
		I	< 8	< 8	< 8	< 8
	HI	VII ₃	8	64	32	128
		I	8	32	32	128

*1 SN antibody titers were examined 3 weeks after the preliminary inoculations. Titers of CF and HI antibodies were observed at the time of challenge.

*2 II and VII₃=isolates II and VII₃, I=smallpox-vaccinia "Ikeda", U=cowpox "Umeno", D=cowpox "Downie"

cows were previously infected with the original and experimental lesions and with smallpox-vaccinia. The animals were then challenged with these materials and 2 strains of cowpox virus. Some of the lesions which appeared on the skin of experimental cow are shown in Photos 7 and 8.

The result seems to suggest that cows preliminary exposed to the original or experimentally reproduced lesions were protected from infections of the homologous materials and cowpox "Downie" more effectively than from smallpox-vaccinia and cowpox "Umeno".

The reaction of humoral antibodies observed in these experimental cows was as shown in Table 4. After the preliminary inoculation, no significant increase in titers of serum-neutralizing (SN) and complement-fixing (CF) antibodies was demonstrated in the cows. On the other hand, an increase in the titer of hemagglutination-inhibiting (HI) antibody against isolates and pox viruses was observed in some of the cows. An increase in HI antibody titer of experimental cow No. 4 was especially remarkable.

The antibody titers observed in serum samples obtained from cows naturally infected during the outbreak of the cowpox-like disease is worthy of note for comparing the serological findings in the experimental cows mentioned above. As previously described⁴¹⁾, no considerable increase in titer of SN antibody against the isolates and control pox viruses was demonstrated in these naturally infected cows. The CF antibody titer of higher than 1:8 against the viruses was not detected in 15 serum samples obtained from the affected cows. Similarly, the titer of HI antibody, which exceeded 1:16, was not observed in the 15 sera tested. On the other hand, a titer of up to 1:16 was observed in the serum of clinically normal cows.

Serum-Neutralization Test

Initially, cross neutralization test of cloned isolates and control pox viruses was performed using convalescent sera of isolates II, VII₃ and cowpox "Downie", and with immune sera against vaccinia and cowpox "Umeno". These antisera were prepared in rabbits as shown in Table 1.

Results of the test, as shown in Table 5, revealed close immunogenicity of these viruses. In addition, it was shown that vaccinal antibody neutralized heterologous viruses with the same or to a higher degree. On the other hand, antibodies against the isolates neutralized these isolates and cowpox "Downie" with the same degree. Especially, neutralization of this cowpox strain with antibodies of both isolates was noticeable. However, antibody against cowpox "Downie" did not neutralize heterologous viruses to the same level as that neutralized homologous virus.

Similarly, as shown in Table 6, the cross SN test by the use of hyperimmunized

Table 5. Result of Cross Neutralization Test of Isolates and Control Pox Viruses with Convalescent or Immune Sera against the Viruses

Viruses	Antisera				
	II Convalescent	VII ₃ Convalescent	IKEDA Immune	UMENO Immune	DOWNIE Convalescent
Isolate II	270(1.00)*	200(1.25)	240(1.33)	41(0.28)	27(0.31)
Isolate VII ₃	150(0.56)	160(1.00)	170(0.94)	15(0.13)	21(0.24)
Smallpox-vaccinia "Ikeda"	77(0.27)	83(0.52)	180(1.00)	32(0.22)	19(0.22)
Cowpox "Umeno"	60(0.22)	28(0.18)	200(1.12)	145(1.00)	40(0.46)
Cowpox "Downie"	430(1.59)	420(2.62)	350(1.94)	100(1.69)	87(1.00)

* Antiserum titer expressed as 50% neutralization end point against 100 TCID₅₀ virus.

The number in parentheses was calculated by $\frac{\text{Ht (Heterologous titer with another virus)}}{\text{Ho (Homologous titer with its own virus)}}$

Table 6. Result of Cross Neutralization Test of Isolates and Control Pox Viruses with Hyperimmunized Antisera

Viruses	Hyperimmunized Antisera				
	II	VII ₃	IKEDA	UMENO	DOWNIE
Isolate II	660(1.00)*	260(1.13)	370(1.19)	160(0.95)	2200(0.48)
Isolate VII ₃	600(0.91)	230(1.00)	380(1.22)	160(0.95)	1800(0.39)
Smallpox-vaccinia "IKEDA"	650(0.98)	250(1.09)	310(1.00)	180(1.05)	2300(0.50)
Cowpox "Umeno"	660(1.00)	190(0.83)	270(0.87)	170(1.00)	2220(0.48)
Cowpox "Downie"	670(1.01)	250(1.09)	170(0.55)	130(0.81)	4600(1.00)

* Refer to Table 5.

Table 7. Result of Cross Complement-Fixation Test of Isolates and Control Pox Viruses with Convalescent or Immune Sera against the Viruses

Antisera Antigens	II Convalescent								VII ₃ Convalescent								Ikeda Immune								Downie Convalescent							
	10	20	40	80	160	320	—	10	20	40	80	160	320	—	10	20	40	80	160	320	—	10	20	40	80	160	320	—				
II	10*						2 0							3 1 0							3 0 0						3 2 0					
	20						2 0							3 0 0							1 0						2 0					
	40						2 0							2 0 0							2 0						2 0					
	80						3 0						3 1 0 0								2 0						3 1 0					
	160						2 0						3 1 0 0								2 1 0						3 2 1 0					
—	3	3	2	1	0	0	0	2	1	0	0	0	0	0	2	1	0	0	0	0	0	2	2	0	0	0	0	0				
VII ₃	10						3 0							2 0 0							1 0						2 0					
	20						3 0							2 0 0							2 0						2 0					
	40						2 0							2 0 0							2 0						1 0					
	80						3 0						2 1 0 0								2 1 0						3 2 0 0					
	160						3 0						3 2 1 0 0 0								3 2 1 0 0					3 2 0 0 0						
—	2	1	1	0	0	0	0	2	1	0	0	0	0	0	2	1	0	0	0	0	0	2	2	1	0	0	0	0				
Smallpox- vaccinia "Ikeda"	10						3 0							3 2 0							2 0						3 0					
	20						3 0							3 1 0							2 0						3 0					
	40						3 0							3 1 0							3 0						3 0					
	80						3 0							3 1 0							3 0						3 0					
	160						3 0							3 2 1 0							3 2 0						3 3 1 0					
—	2	2	2	1	0	0	0	2	1	0	0	0	0	0	3	2	0	0	0	0	0	2	2	0	0	0	0	0				
Cowpox "Downie"	10						3 0							3 1 0							3 1 0						2 0					
	20						3 0							3 1 0							2 1 0						3 1 0					
	40						3 0							3 1 0 0							3 2 1 0 0						3 2 2 0 0					
	80						3 0							3 2 1 0 0							3 2 1 1 0 0						3 3 2 1 1 0 0					
	160						3 0							3 3 2 1 0 0 0							3 2 1 0 0 0					2 2 1 1 1 0 0						
—	3	2	2	0	0	0	0	2	2	1	0	0	0	0	3	2	1	0	0	0	0	2	2	1	0	0	0	0				

* Dilution of antigens or antisera. □ Zone of anticomplementary action of antisera.

▨ Zone of complete fixation. 3: nearly complete, 2: incomplete, 1: trace, and 0: none fixation of the complement.

antisera revealed no marked difference in immunogenicity of the viruses. However, a slight difference in immunogenicity of cowpox "Downie" was again noted in this test.

Employing hyperimmunized rabbit sera and culture fluid of HeLa cells infected with cloned viruses, adsorption of antibody to homologous viruses was conducted, in order to differentiate the viruses. However, the antiserum was diluted over 8 times by the addition of the virus material even in the most efficient case. In the most inefficient case, such a combination as low titer of virus with high titer of the serum, it was presumed that the serum should be diluted over 20 times in order to obtain complete adsorption. From this result, differentiation of the viruses by the use of adsorbed serum was not attempted.

Complement-Fixation Test

Cross complement-fixation test of isolates and control pox viruses was conducted with their convalescent or immune sera which were used in the SN test. As shown in Table 7, in this test, anticomplementary action of the sera was observed with all of them at dilutions

Table 8. Result of Cross Complement-Fixation Test of Isolates and Control Pox Viruses with Hyperimmunized Antisera

Antigens	Hyperimmunized Antisera				
	II	VII ₃	IKEDA	UMENO	DOWNIE
Isolate II	64*	16	64	32	128
Isolate VII ₃	128	16	64	32	128
Smallpox-vaccinia "Ikeda"	128	16	64	64	128
Cowpox "Umeno"	64	16	64	64	128
Cowpox "Downie"	64	16	64	32	128

* Dilution of antiserum, end point of complete fixation.

Table 9. Result of Cross Hemagglutination-Inhibition Test of Isolates and Control Pox Viruses with Hyperimmunized Antisera

Viruses	Hyperimmunized Antisera				
	II	VII ₃	IKEDA	UMENO	DOWNIE
Isolate II	2048*	512	4096	2048	256
Isolate VII ₃	1024	512	4096	1024	128
Smallpox-vaccinia "Ikeda"	2048	2048	8192	8192	512
Cowpox "Umeno"	2048	1024	4096	4096	512
Cowpox "Downie"	1024	1024	4096	4096	1024

* Dilution of antiserum, end point of complete inhibition of hemagglutination.

lower than 1 : 20, and the action of convalescent serum of isolate II was still maintained in dilution of 1 : 80. However, zones of complement-fixation with the antigens and antisera were approximately the same irrespective of their homologousness, indicating close antigenicity of these viruses.

Similarly, cross CF test by the use of hyperimmunized antisera showed similarity in antigenicity of these viruses, as shown in Table 8.

Hemagglutination-Inhibition Test

Result obtained in cross hemagglutination-inhibition test of cloned isolates and control pox viruses with their hyperimmunized rabbit antisera is shown in Table 9.

No marked differences among immunogenicity of the viruses were revealed. However, HI titers of antiserum against cowpox "Downie", which were observed with isolates II and VII₃ (1 : 256 and 1 : 128), seemed slightly lower than those observed with homologous virus (1 : 1024) and the other pox viruses (1 : 512).

Gel Diffusion Test

Table 10 shows the result of cross gel diffusion test, in tubes, of antigens, prepared from isolates and control pox viruses, with their homologous and heterologous hyperimmunized rabbit antisera.

Precipitation fractions formed by the reaction of antigens and their antisera were relatively small in number, comparing to the result obtained in the gel diffusion test of pox viruses by GISPEN¹⁷⁾, and RONDLE et al.³⁸⁾ However, from the result obtained in our test, the antigenicity of the two isolated strains and smallpox-vaccinia "Ikeda" and cowpox "Umeno" was identical. The slight difference observed with the use of antigen prepared from the isolate VII₃ is of interest.

On the other hand, the antigen of cowpox "Downie" seemed to be distinguishable from

Table 10. Result of Cross Gel Diffusion Test of Isolates and Control Pox Viruses with Hyperimmunized Antisera

Antigens	Hyperimmunized Antisera				
	II	VII ₃	IKEDA	UMENO	DOWNIE
Isolate II	I,*2,(3)	1,2,3	1,2,3	1,2,3 or (III),4	1,3
Isolate VII ₃	1,2,3	I or (I'),2,3	I or (I'),II,(3)	1,2,3 or (III),4	(1'),2,3
Smallpox-vaccinia "Ikeda"	1,2,3	1,2,3	1,2,3	1,2,3,4	1,3
Cowpox "Umeno"	1,2,3	1,2,3	1,2,3	1,2,3,4	1,3
Cowpox "Downie"	2	(2)	2,3'	1',3',(4)	2,3

* Precipitation fractions were numbered in the order they appeared from top to bottom of an agar column. Roman numerals indicate major and distinct precipitation fractions, whereas Arabic numerals show fractions which were faint and sometimes disappeared. Numbers with the mark, ' show fractions which did not completely fit the fractions numbered without the mark. Numbers in parenthesis indicate the fraction which appeared occasionally.

the other antigens by the demonstration of one or two precipitation fractions which were not similar to those produced by the remaining antigens. However, it seemed that there was a slight difference between the precipitation fractions formed by the reaction of antisera of the isolates and those produced by antisera of smallpox-vaccinia "Ikeda" and cowpox "Umeno" against antigen "Downie".

Further tests using agar gel diffusion of these viruses are necessary.

DISCUSSION

On the difficulty in isolation of causative agents of the outbreak

The most noticeable feature experienced during the investigations on cowpox-like disease outbreak in Hokkaido, Japan, was the difficulty involved during the isolation of the causative agents. Attempts made to isolate the agents from skin lesions of naturally infected cows were repeated without success, employing skin, testicles of rabbit, CAM of chicken embryonating egg, and cell cultures. First of all, the isolation of the agent from skin lesions of experimental cows and rabbits on the CAM was entirely negative. Similar results obtained in isolation experiment on cowpox-like disease were reported by HESTER et al.²¹⁾, AKAZAWA¹⁾, and MOTOYOSHI et al.³³⁾ WHEELER et al.⁴⁶⁾ also reported negative isolation result of viral agent from a patient having milker's nodules (According to GOTTRON¹⁸⁾, BECKER²⁾, and other investigators, it seems impossible to clinically differentiate between cowpox and milker's nodules; thus the discussion will be directed, for a while, to involve both diseases into one entity.). KATZENELLENBOGEN²⁶⁾ succeeded in producing nodules on rabbit skin 6 days after the inoculation of fresh lesion obtained from a cow suffering from milker's nodules. The nodule obtained from a rabbit was successfully transmitted to another rabbit. In further test, the investigator²⁷⁾ found intracellular inclusion bodies in the lesional material, but the isolation of viral agent from the infected cow was unsuccessful. From the results, KATZENELLENBOGEN presumed the disease as paravaccinia. BECKER²⁾, who attempted to isolate the causative agent from a man infected with milker's nodules, reported negative result. He concluded that the causative agent of the disease can be closely associated with paravaccinia virus.

NASEMAN et al.³⁴⁾ indicated that virus of paravaccinia is the same as that of genuine milker's nodules. According to their investigations, paravaccinia virus was negative in Paul's test and cultivation on the CAM, but it was capable of producing inclusion bodies in the nucleus and cytoplasm of the epithelial cells of a skin lesion. Particles of the virus, as revealed by electron microscope, were slenderer and longer than those of vaccinia virus.

Recently, FRIEDMAN-KIEN et al.¹⁶⁾ isolated pox virus from a patient having milker's nodules on the primary cultures of bovine kidney. More recently, MOSCOVICI et al.³²⁾ isolated similar virus on the primary cultures of fetal bovine testicles. Both of these viruses did not produce pock lesions on the CAM nor were immunologically related to vaccinia virus. However, PAIT³⁵⁾ censured for the lack of evidence that these isolates are actually the causative agents of the disease in question.

On the contrary, vaccinia virus was easily isolated by SCHULTZE et al.⁴⁰⁾ from nodes obtained from a patient of milker's nodules, by LEROY et al.²⁹⁾ from serous fluid of pustular lesion of patients infected with cowpox, and by HUYGELEN et al.^{23,24)} from a lesional skin and oral ulcers of an infected cow. However, the isolation of vaccinia virus from milker's nodules was not always easy as those described. ZURUKZOGLU et al.⁴⁸⁾ finally obtained an agent from a pustule, characteristic of vaccinia infection, after five successive passages of the content of milker's nodules through rabbit skin.

At the early stage of our investigation, the disease seemed to be caused by milker's nodule virus (paravaccinia, *Strongyloplasma paravaccinae*), because the causative agent could not be isolated in spite of the presence of inclusion and elemental bodies in the skin lesion of cow or rabbit. In addition, the clinical finding in a cow, which is characterized by non-febrile reaction, smaller pustules (Photo 9) with a tendency to spread as compared to the case when vaccinia virus was artificially inoculated, and very slight protection against vaccinia infection³⁾, supported our conjecture. However, as described in the previous report, viral agents were finally isolated from infected rabbit testicles on the CAM and HeLa cells, and from infected rabbit skin on HeLa cells. The properties of these isolates were not differentiable from those of vaccinia virus. It is interesting to note that in order to isolate these viruses a serial passage through rabbit testicles was necessary; HORGAN²²⁾ employed serial passage through rabbit testicles to vaccinate the variola virus.

How the isolates be named?

Etymologically, vaccinia virus is believed to have originated from *res vaccina*, i. e., as belonging to a cow (*vacca*). Accordingly, vaccinia virus actually means cowpox virus. However, the term, vaccinia virus has changed to signify a strain of variola or other pox virus which is adapted to cattle, or it merely means laboratory strain (*Poxvirus officinale*). Especially, since Downie's description¹⁰⁻¹²⁾, both viruses, cowpox and vaccinia, have been differentiated from each other by VERLINDE⁴⁵⁾, GISPEN¹⁷⁾, BERGER⁴⁾, MORITSCH³¹⁾, FENNER et al.¹⁵⁾, RONDLE et al.³⁸⁾, and KATO et al.²⁵⁾, on the basis of cytoplasmic inclusion bodies, histological reaction, serological observations, including gel diffusion, and findings on the infected CAM.

DIXON⁸⁾ stated in his monograph that cowpox is not a natural disease of bovines. In Jenner's day the disease was believed to have originated from smallpox virus, whereas presently the infection is attributed to vaccinia virus, either by direct passage, or after passage through bovines or other animals. It then assumes the "wild" properties of *Poxvirus bovis*. On the other hand, HERRLICH et al.²⁰⁾ pointed out that vaccinia virus was not derived from variola virus but it is the original independent member of the pox group of natural occurrence. The virus is widespread among animals and many cases of animal pox disease are caused by it. This explanation is based on their negative result obtained in trials of transformation of variola or alastrum virus to vaccinia virus.

In our investigations, it was difficult to differentiate between the skin lesions in cattle produced by inoculations of vaccinia "Ikeda" or our isolates and the cowpox "Downie" (Photos 10, 11, 12 and 13). Slight differences in the appearance of lesions, delayed incubation period and rapid development to scab after the pock formation were observed with the cowpox virus strain. According to DOWNIE¹⁴⁾, DEKKING isolated cowpox virus from 28 cows and vaccinia virus from 8 out of a total of 36 cows, which were clinically diagnosed as

having cowpox. In an experimental infection of cattle with cowpox virus conducted by BERGER et al.⁵⁾ and PUNTTIGAM et al.³⁷⁾, the haemorrhagic lesions, which are usually produced on rabbit skin by the virus, were not observed. These results suggest the difficulty involved in the differential diagnosis of infections in cattle with viruses of cowpox and vaccinia.

For the differentiation of infections due to the both viruses, the "A" type inclusion body has been pointed out by KATO et al.²⁵⁾ to be significant. In our investigations, this type of inclusion body was demonstrated in the skin lesions of experimental cow, rabbits, and so forth as described elsewhere⁴¹⁾. However, these inclusion bodies were not demonstrated in infected cell cultures derived from several animal species. The "A" type inclusion bodies were found by TAKAHATA⁴²⁾ in the epithelial cell of a cornea or skin of his experimental cows or rabbits inoculated with the skin lesions obtained from a naturally infected cow. According to BERGER⁴⁾, cowpox virus initially produced large inclusion bodies in skin of cows, but the ability to produce the large inclusion bodies was lost when the virus was transferred to a host other than cattle. DOSCH et al.⁹⁾ demonstrated a reversible occurrence of inclusion body of cowpox virus. TONGEREN⁴³⁾ showed that a white pock, which was branched off from cowpox virus, produced smaller inclusion bodies, in size, upon progressive passages. The virus changed in pathogenicity for mouse, and it showed properties closer to a vaccinia virus. Thus, the type of inclusion body does not always seem to be applicable for the identification of virus.

However, cowpox "Downie", which was employed as one of the known control pox viruses in our investigations, has never changed in its reproducibility of the hyaline type inclusion bodies. The virus was easily recovered, in HeLa cell cultures, from a lesional skin of cow which was experimentally exposed to the infected CAM. If such a strain as cowpox "Downie" is easily isolated from skin lesions of naturally infected cattle, our isolates can not be classed as cowpox virus. Accordingly, it is presumable that our isolates are vaccinia virus of low virulence (GOTTRON¹⁸⁾) and the difficulty experienced in the isolation experiment is owed to biological modification (ZURUKZOGLU et al.⁴⁸⁾).

On the vaccinal contamination through the isolation experiment

Smallpox vaccine has been prepared in this laboratory employing vaccinia "Ikeda". Therefore, careful attention was paid in avoiding contamination of the virus.

If contamination is possible, it can occur during the stage of passages on experimental cattle or rabbit. However, experimental cattle were maintained in a new barn consisting of 4 isolated pens which were similar to those constructed by J. A. BAKER in Veterinary Virus Research Institute, Cornell University, New York, U. S. A. Until our isolation experiment was started, the barn had been closed for about 10 months since its construction. During the course of the experiment, none of the known control pox viruses was brought into the barn until the causative agents were successfully isolated on CAM. If the lesional skin of experimental cattle was produced as a result of contamination of the vaccinia virus, the virus can be easily recovered on CAM or HeLa cell cultures. However, the isolation of the causative agent from lesional skin of cattle at the 1, 2 and 3 pass-levels was not successful on the CAM and HeLa cells. Contrarily, the lesional skin of an experimental cow produced by pocks on the CAM which were formed by our isolate facilitated the recovery on the hosts. In addition to this, lesions were not produced on the skin of these experimental cattle, with physiological saline containing penicillin and streptomycin and with suspension of *Staph. albus* isolated from the original skin lesion. From these findings the spontaneous contamination of vaccinia virus in experimental cattle seems to be deniable.

Similarly, contamination of the vaccinia virus in rabbits could be neglected by the difficulty in isolation.

It is acknowledged that the isolation of the causative agent from experimental skin lesions of cattle and rabbit was unsuccessful; even inclusion and elemental bodies were found in the

lesions. For example, as shown in Photo 14, many of the inclusion bodies were recognized in weak skin lesion (Photo 15) which was experimentally produced on the teat of a cow after inoculation of the original skin lesion labelled as XVI. Also when lesion was examined by electron microscope, developmental forms of pox virus were revealed in the epithelial cells and mature particles mostly in the horny layer (Photo 16). The present authors believe that the isolates were derived from these viruses and not from a contaminant.

On the non-reproducibility of the experiment

Cow skin lesions, at the 2nd pass-level, labelled as II and as VII₃, and which initially produced pustular lesions on rabbit skin, were re-tested on rabbit without success. In this re-test, rabbits received the 2 specimens of the lesional skin showed pustular lesions. Then these pustular lesions were successively passed through rabbit testicles. A marked swelling of the testicles was observed at the 1st and 2nd pass-levels but not at the 3rd and 4th pass-levels. Pocks on the CAM were not produced with these skin and testicles of rabbits. The cow skin lesions, employed in these tests, were initially tested after storage at -20°C for 24 hours (harvested on Nov. 21, 1962) and re-tested after a storage for 142 days.

Negative isolation experiment was also experienced with cowpox-like disease which occurred in 1963, in Urawa City, Japan. The occurrence of this disease was firstly recognized on Feb. 5, 1963 and a total of 5 skin lesions were obtained from 3 cattle and 2 men on the 14th of February. These lesions were inoculated dermally into an experimental cow on March 4th. Positive take was observed using 2 lesions obtained from cow. These experimental lesions were then successively transferred to the skin and testicles of rabbit and to the CAM without success.

The failure to produce skin lesion on experimental cattle by inoculation of natural lesions of cow was reported by HESTER et al.²¹⁾ and MOTOYOSHI et al.³⁸⁾ ZURUKZOGLU et al.⁴⁸⁾ once succeeded in isolating the causative agent of milker's nodules, which were obtained from a man, by four successive passages through rabbit. However, the lesion obtained from the same man on the following day did not give a positive result, even the lesion was transferred through six successive passages on rabbit. As previously reported⁴¹⁾, positive take was observed with 3 samples on Exp. cow No. 1 and 5 samples on Exp. cow No. 2 using a total of 17 samples of lesional skin harvested from cows affected in the outbreak of the disease in Hokkaido. Isolation of the causative agents was successful by serial passages through cow and rabbit, employing these positive lesions as starting materials. In general, the so-called typical lesion was intended for isolation experiment. However, it should be ascertained as to whether the manner is the most suitable or not.

A marked decrease in virus titer of a 10% suspension of CAM infected with isolate II was observed upon the storage at -20°C . Prior to the storage, the suspension of the CAM showed titer of 1.8×10^8 pock forming units per ml, and it dropped to 1.5×10^8 after the storage for 21 months. In the isolation of smallpox virus from the blood of a patient suffering from smallpox, DOWNIE et al.¹³⁾ pointed out a marked loss of virus in the blood after a few weeks of refrigeration. These findings suggest that the length of storage period of test material has an influence on the recovery of the infective virus.

If the investigations on cowpox or milker's nodules always resulted in reproducibility by any one anywhere, continued controversy in regards to the disease is not necessary for over one-half century following the first report of WINTERNITZ⁴⁷⁾ in 1899. On this ground the present authors wish to publish this report.

Nomenclature of the disease

It will be appropriate to designate the cowpox-like disease on the basis of clinical symptoms caused by an infection of the isolates and their viral properties.

According to HERRLICH et al.¹⁹⁾ diseases of cattle which require differentiation from cowpox are stomatitis papulosa, stomatitis vesicularis, pseudoaptha stomatitis and foot-and-

mouth disease. However, in such a case when localized lesions occur on the teats and udder, as we experienced, differentiation from milker's nodules will be the most important. Many of detailed descriptions have been presented on infection of milker's nodules in man. According to the descriptions, infection of milker's nodules is characterized by the formation of nodules and lack of immunity against smallpox infection. Contrarily, lesions due to cowpox infection develop into pustules, and the infection gives the host immunity to smallpox. However, reports of clinical observations made on milker's nodules infection in cattle are scarce. In a total of 9 reports on cattle which were regarded as the source of milker's nodules in man, diagnosed cases of cowpox or typical cowpox appeared in 5 reports. This indicates the existence of practical difficulty in differential diagnosis of cowpox and milker's nodules in cattle. From these findings mentioned above, the disease of cattle experienced in Hokkaido can be diagnosed clinically as cowpox, even though clinical findings in man affected with the disease are close in some respects to those observed in infection of milker's nodules.

The isolation of the causative agent from materials obtained from cattle infected with cowpox, seems easy by using the conventional procedure, in either cases of vaccinia virus (SCHULTZE et al.⁴⁰⁾) or cowpox virus (DEKKING⁷⁾, and DAVIS⁶⁾). However, in case of milker's nodules infection, isolation of the causative agent is only occasionally successful. If the difficulty experienced in obtaining our isolates is allowed to be taken into consideration, the disease of cattle is not distinguishable from milker's nodules. However, through comparison of our isolates to vaccinia and cowpox viruses, it was demonstrated that the properties of a majority of the isolates is closer to those of vaccinia virus than those of cowpox "Downie".

Name of the disease, cowpox, is termed hitherto on the basis of clinical finding in cattle, and it is not based on its causative agent. The causative agent of a contagious disease which is presently considered as different from cowpox disease may be considered as the cause of cowpox disease, in addition to viruses of vaccinia and cowpox.

Thus, the cowpox disease of today can be assumed as a clinical syndrom or entity which is due to an infection by various pox group of viruses. Consequently, the outbreak of cowpox-like disease investigated by the present authors have been termed as cowpox disease.

SUMMARY

The properties of viral agents, isolated during the cowpox-like disease outbreak in Hokkaido, Japan, fall of 1962, were investigated by comparing them with vaccinia and cowpox viruses.

The agent which was isolated with difficulty on CAM of chicken embryonating eggs and HeLa cell cultures, were identical to vaccinia virus in several respects, but it was not similar to cowpox virus "Downie" (LB red).

The disease of cattle was diagnosed as cowpox from the standpoint of a disease which showed clinical syndrom or entity in cattle affected with pox group of viruses.

REFERENCES

- 1) AKAZAWA, S. (1952): *Bull. Nippon Vet. Zootechn. Coll.*, **1**, 1~9.
- 2) BECKER, FR. T. (1940): *J. Amer. Med. Ass.*, **115**, 2140~2144.
- 3) BERGER, K. (1955): *Zbl. Bakt., I, (Orig.)*, **162**, 363~372.
- 4) BERGER, K. (1956): *Z. Hyg.*, **143**, 151~158.
- 5) BERGER, K. and PUNTIGAM, F. (1958): *Zbl. Bakt., I, (Orig.)*, **172**, 363~369.
- 6) DAVIS, J. H. T., JANES, L. R. and DOWNIE, A. W. (1938): *Lancet*, *ii*, 1534~1538.
- 7) DEKKING, F. (1950): *Tijdschr. Diergeneesk.*, **75**, 248~250.

- 8) DIXON, C. W. (1962): Smallpox, London, J. & A. Churchill.
- 9) DOSCH, F. and MORITSCH, H. (1956): *Zbl. Bakt., I, (Orig.)*, **166**, 517~528.
- 10) DOWNIE, A. W. (1939): *J. Path. & Bact.*, **48**, 361~379.
- 11) DOWNIE, A. W. (1939): *Brit. J. Exp. Path.*, **20**, 158~176.
- 12) DOWNIE, A. W. and HADDOCK, D. W. (1952): *Lancet*, **ii**, 1049~1050.
- 13) DOWNIE, A. W., MCCARTHY, K., MACDONALD, A., MACCALLUM, F. O. and MACRAE, A. D. (1953): *Lancet*, **265**, 164~166.
- 14) DOWNIE, A. W. and DUMBELL, K. R. (1956): *Ann. Rev. Microbiol.*, **10**, 237~252.
- 15) FENNER, FR. (1958): *Virology*, **5**, 502~529.
- 16) FRIEDMAN-KIEN, AL. B., ROWE, W. P. and BANFIELD, W. G. (1963): *Science*, **140**, 1335~1336.
- 17) GISPEN, R. (1955): *J. Immunol.*, **74**, 134~141.
- 18) GOTTRON, H. (1930): *Derm. Z.*, **58**, 207~221.
- 19) HERRLICH, A., MAYR, A. and RODENWALDT, E. (1960): Die Pocken, Stuttgart, Georg Thieme Verlag.
- 20) HERRLICH, A., MAYR, A., MAHNEL, H. and MUNZ, E. (1963): *Arch. Ges. Virusforsch.*, **12**, 579~599.
- 21) HESTER, H. R., BOLEY, L. E. and GRAHAM, R. (1941): *Cornell Vet.*, **31**, 360~378.
- 22) HORGAN, E. S. (1938): *J. Hyg.*, **38**, 702~715.
- 23) HUYGELEN, C., MORTELMANS, J., THIENPONT, D., BICHE, Y. and PINCKERS, F. (1958): *Zbl. Vet.-Med.*, **5**, 859~870.
- 24) HUYGELEN, C., TONGEREN, H. A. E. VAN and MORTELMANS, J. (1959): *Zbl. Vet.-Med.*, **6**, 154~161.
- 25) KATO, SH. and KAMAHORA, J. (1962): *Symp. Cell. Chem.*, **12**, 47~90.
- 26) KATZENELLENBOGEN, I. (1935): *Acta Derm.-Venereol.*, **16**, 316~327.
- 27) KATZENELLENBOGEN, I. (1952): *Dermatologica*, **105**, 69~78.
- 28) KITAOKA, M., TAGAYA, I., OZAWA, Y., SUZUKI, M. and OBARA, M. (1953): *Jap. J. Bakt.*, **8**, 605~609.
- 29) LEROY, D., BIZAIS, J., RICHIER-CHEVREL, M.-E. and RICHIER, J.-L. (1952): *Bull. Acad. Nat. Med.*, **136**, 546~549.
- 30) MAYOR, H. D., JAMISON, R. M. and JORDAN, L. E. (1963): *Virology*, **19**, 359~366.
- 31) MORITSCH, H. (1957): *Z. Tropenmed. Parasit.*, **8**, 333~348.
- 32) MOSCOVICI, C., COHEN, E. P., SANDERS, J. and DELONG, S. S. (1963): *Science*, **141**, 915~916.
- 33) MOTOYOSHI, S., SATO, K. and OKADA, K. (1963): *J. Vet. Med.*, No. 351, 14~18.
- 34) NASEMAN, TH. and BAUER, E. (1957): *Klin. W.*, **35**, 62~65.
- 35) PAIT, CH. F. (1963): *Science*, **142**, 1422~1424.
- 36) PLOTZ, H. (1942): *Science*, **95**, 441~442.
- 37) PUNTIGAM, F. and BERGER, K. (1958): *Z. Hyg.*, **145**, 199~208.
- 38) RONDLE, C. J. M. and DUNBELL, K. R. (1962): *J. Hyg.*, **60**, 41~49.
- 39) SATO, Y. (1956): *Virus*, **6**, 429~435.
- 40) SCHULTZE, W., SEIFRIED, O. and SCHAAF, J. (1927): *Z. Infekr.*, **31**, 295~313.
- 41) SOEKAWA, M., MATSUMOTO, K., IZAWA, H., IWABUCHI, H., TAKAHASHI, K. and SAITO, Y. (1964): *Jap. J. Vet. Sci.*, **26**, 25~41.
- 42) TAKAHATA, K. (1963): *J. Hokkaido Vet. Med. Ass.*, **7**, No. 5, 1~5.
- 43) TONGEREN, H. A. E. VAN (1952): *Arch. Ges. Virusforsch.*, **5**, 35~52.
- 44) UMENO, SH. (1907): *Saikingaku-Zashi*, No. 140, 409~419.
- 45) VERLINDE, J. D. (1951): *Tijdschr. Diergeneesk.*, **76**, 334~342.
- 46) WHEELER, CL. E. and CAWLEY, E. P. (1957): *Arch. Derm. Syph.*, **75**, 249~259.
- 47) WINTERNITZ, R. (1899): *Arch. Derm. Syph.*, **49**, 195. [Cited from (40)]
- 48) ZURUKZOGLU, ST. and KUSKE, E. (1938): *Acta Derm.-Venereol.*, **19**, 569~586.

北海道に発生した牛痘様疾患について

II. 分離ウイルスの性状ならびに病名

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我々は研究の初期には、野外材料から CAM への病原分離が極めて困難であり、試験接種牛および家兎の皮膚発痘が vaccinia virus 感染のそれにくらべて小さく且つ周囲に拡がる傾向があり、これらの発痘部から CAM にウイルスの分離が出来ず、さらに試験牛の交叉防禦試験の成績等から見て所謂 *Strongyloplasma paravaccinae*^{2,26,27,34)} のようなものが

原因かと考えた。しかし前報で述べた経過を辿り HeLa 細胞または CAM にとり出したウイルスの諸性状は、本文に詳述したように vaccinia virus (*Poxvirus officinale*) と区別出来難いものである。

临床上牛痘所見を呈したもののから痘瘡群ウイルスを分離しえたので本症を牛痘と称ぶことにする。

EXPLANATION OF PLATES

PLATE I

- Photo 1. Virus particles of isolate II grown and cloned in HeLa cell cultures. Purified with fluorocarbon and negatively stained with phosphotungstate; $\times 120,000$.
- Photo 2. Virus particles as revealed in thin section of the CAM of chicken embryonating egg which was finally obtained by serial passage of the original cow skin lesion II through cow skin (2 successive passages), rabbit skin and testicles; $\times 27,000$.
- Photo 3. Virus particles as observed in thin section of the cornea of a rabbit infected with the CAM which was obtained by 3 additional successive passages of the lesional CAM mentioned in Photo 2. The section was prepared from the cornea on the 3rd day of infection; $\times 15,000$.

PLATE II

- Photo 4. Cytoplasmic inclusion bodies of Guarnieri type as observed in the primary monolayer cells of goat kidney infected with isolate II. Stained with Giemsa after methanol fixation, 48 hours after infection.
- Photo 5. Cytoplasmic inclusion bodies of the hyaline type as revealed in the primary monolayer cells of goat kidney infected with cowpox "Downie". Stained with Giemsa after methanol fixation, 48 hours after infection.
- Photo 6. Cytoplasmic inclusion bodies of Guarnieri type as they appeared in smear preparation from an infected rabbit cornea. This smear preparation was obtained from the same corneal sample as mentioned in Photo 3. Stained with Giemsa after methanol fixation.

PLATE III

- Photo 7. Lesions produced by challenge of isolate VII₃ and cowpox "Umeno" on the skin of the anterior left flank of Exp. cow No. 3 which was previously infected with the original cow skin lesions. Seven days after challenge. Take is negative with the isolate (VII) and moderate with the cowpox virus (CU). $\times 10$ and $\times 50$ indicate dilution of inocula.
- Photo 8. Lesions produced by challenge viruses of vaccinia "Ikeda" and cowpox "Downie" on the skin of the posterior left flank of Exp. cow No. 3 which was previously infected with the original cow skin lesion. Seven days after challenge. Moderate take with the vaccinia virus (V) and negative take with the cowpox virus (CD) are shown. $\times 10$ and $\times 50$ indicate dilution of inocula.

PLATE IV

- Photo 9. A lesion appeared on the teat of Exp. cow No. 5 after a dermal inoculation of the original cow skin lesion VII₃. Five days after inoculation.
- Photo 10. A lesion produced by dermal inoculation of isolate II on the teat of Exp. cow No. 5. Five days after inoculation.
- Photo 11. A lesion on the skin of Exp. cow No. 5 produced by dermal inoculation of isolate II. Five days after inoculation.
- Photo 12. A lesion on the teat of Exp. cow No. 5 produced by dermal inoculation of cowpox "Downie". Ten days after inoculation.
- Photo 13. Skin lesion of Exp. cow No. 5 produced by dermal inoculation of cowpox "Downie". Five days after inoculation. Delayed development of lesion as compared to that produced by isolate II (Photo 11), is worthy of note.

PLATE V

- Photo 14. Cytoplasmic inclusion bodies observed as in a section prepared from a lesion observed on the base of the teat of Exp. cow No. 2 produced by the original cow skin lesion XVI. Photo 15 illustrates this lesion. The lesion was harvested 5 days after inoculation for histopathological examination. Stained with H. E. after formol fixation; $\times 550$.
- Photo 15. Skin lesion produced by dermal inoculation of the original cow skin lesion XVI on the base of the teat of Exp. cow No. 2. Five days after inoculation.

- Photo 16. Virus particles as revealed by electron microscope in a thin section prepared from an experimental lesion produced on the teat of a cow which is illustrated in Photo 15. This thin section was prepared from the section which is illustrated in Photo 14.