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Preparation of Monoclonal Antibodies Cross-Reactive with Orthopoxviruses and Their Application for Direct Immunofluorescence Test

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***Abstract*: Variola virus (smallpox virus), vaccinia virus (VV), cowpox virus (CPV) and ectromelia virus (EV) belong to the genus *Orthopoxvirus* of the family *Poxviridae*. To establish the possible diagnosis for smallpox infection, monoclonal antibodies (MAbs) against VV and CPV were produced. The cross-reactivity of seven MAbs with cells infected with various strains of the orthopoxviruses (CPV, VV and EV) was confirmed by an immunofluorescence (IF) test and other immunological analyses. Four and three MAbs reacted with the common antigen of all poxviruses (probably NP antigen) and the antigen involved in neutralization, respectively. We developed the IF test using these MAbs. The direct IF test required only 45 min to perform. Smallpox infection is now eradicated, but it is important to prepare for the diagnosis of smallpox in an emergency. The direct IF assay using MAbs cross-reactive with orthopoxviruses is rapid, simple, specific, applicable for multiple samples, and will make it possible to screen for and detect orthopoxviruses that include variola virus with tissue impression smears from skin lesions in most laboratories or institutes.**

***Key words*: Monoclonal antibody, Smallpox, Rapid diagnosis, Cross-reactivity**

Smallpox outbreaks have occurred from time to time for thousands of years, but this infectious disease in nature is now eradicated after a successful worldwide vaccination program. The last naturally occurring case in the world was in Somalia in 1977. Variola virus (smallpox virus) caused smallpox infection. Except for laboratory stockpiles, the variola virus has been eliminated. However, there is heightened concern that the variola virus might be used as an agent of bioterrorism or biowarfare. Therefore, the government of each country is taking precautions for dealing with a smallpox outbreak. Smallpox is a serious, contagious, and sometimes fatal disease. Although the only prevention is vaccination, routine vaccination among the general public was stopped. There is no specific treatment for the smallpox disease.

Confirmation of a smallpox outbreak requires diagnosis. Laboratory diagnostic testing for the variola virus has been conducted by serological assays, negative stain electron microscopy (EM) identification, or isolation of the variola virus from a clinical specimen. Recently, diagnosis has been performed by polymerase chain reaction (PCR) (2, 4, 16, 22), restriction fragment length polymorphism (19), microarrays (17) and the sequencing (23) of viral DNA. Genetic diagnosis has a high sensitivity and specificity, but might be costly, complicated and time-consuming. In contrast, the serological assay may be more rapid and simple than the

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Abbreviations: ATI, A-type inclusion body; CPV, cowpox virus; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; EV, ectromelia virus; FITC, fluorescein isothiocyanate; HRPO, horseradish peroxidase; IF, immunofluorescence; IP, immunoprecipitation; MAb, monoclonal antibody; MCV, Molluscum contagiosum virus; MIF, membrane IF; NP, nucleoprotein; NT, neutralization; PCR, polymerase chain reaction; PFU, plaque-forming unit; SFV, Shope fibroma virus; VV, vaccinia virus; WB, western blotting.

genetic diagnosis, EM or isolation of virus, but is not yet practical as a diagnostic tool in most laboratories.

Variola virus, vaccinia virus (VV), cowpox virus (CPV) and ectromelia virus (EV) belong to the genus *Orthopoxvirus* of the family *Poxviridae*. Several major antigens have been shown to be induced in cells infected with CPV and VV, i.e., the nucleoprotein (NP) antigen (24, 29), neutralization (NT)-associated antigen (21) and A-type inclusions (ATI) (7–9). We have identified the polypeptides of the major antigens by immunoprecipitation (IP) with polyclonal antibodies (5, 6) and identified those antigens by IP and immunoblotting (western blotting, WB) with a monoclonal antibody (MAb) (11–13). The NP is a so-called common antigen that shows cross-reactivity with cells infected with either an *Orthopoxvirus* such as CPV, EV and VV, or a *Leporipoxvirus* such as the Shope fibroma virus (SFV). The NT antigen shows cross-reactivity with cells infected with the *Orthopoxvirus* such as CPV, EV and VV. On the other hand, the ATI are seen in cells infected only with an *Orthopoxvirus* such as CPV or EV.

In this study, we newly produced MAbs cross-reacted with the orthopoxviruses, and examined the development of simple, rapid, and reliable diagnostic screening for the multiple tissue impression smears infected with orthopoxviruses that include the smallpox virus.

Materials and Methods

Viruses and cells. RK13 cells derived from rabbit kidney cells, HeLa cells derived from a human carcinoma and L cells derived from mouse fibroblasts were grown in minimum essential medium supplemented with 5% calf serum (MEM-CS). The strains of CPV were the LB red, Amsterdam, 53, 58 and 60 strains, the EV strains were Ishibashi and Hampstead, the VV strains were Ikeda, Lister, IHD-W and IHD-J, and the SFV strain was OA (5, 6, 11–13). The RK13 or HeLa cells were infected with CPV, VV or SFV, and the HeLa or L cells were infected with EV. Tissue samples infected with Molluscum contagiosum virus (MCV) were kindly provided by Dr. Kenji Hiroi (Hiroi Dermatologic Hospital, Osaka, Japan).

Production of hybridoma. The methods for the production and culture of the hybridomas and the screening procedures for the antibodies have been previously described (11). Hybridomas were obtained from the cultures of PAI myeloma cells fused with spleen cells from BALB/c mice immunized with cells infected with the LB red strain of CPV, or Ikeda strain or Lister strain of VV. The obtained ascites fluids as previously described (12) were used as MAbs. The subclasses of these antibodies were determined by immunodiffusion with a hybridoma cell lysate and class-specific immunoglobulin antisera as described by Volk et al. (27).

Immunofluorescence (IF) test. The direct and indirect (IF) test was carried out with cells infected with each virus at 2 to10 PFU (plaque-forming unit)/ml cultured for 24 hr after infection and fixed with cold acetone. For the membrane IF (MIF) test, cells infected and cultured were used without fixation. The FITC (fluorescein isothiocyanate isomer I, Becton Dicknson & Co., U.S.A.)-conjugation with antibody can be performed using the methods of Kawamura et al. (10). Briefly, the antibody (ascites) was concentrated with a saturated solution of ammonium sulfate and dialyzed with 0.85% NaCl buffer (pH 7.0). The FITC was dissolved with 500 mM carbonate-bicarbonate buffer (pH 9.5), added to produce a ratio of 10–40 μg/mg of antibody solution, and immediately mixed. The mixture was incubated and rotated at room temperature for 2 hr. The unreacted FITC or MAb was removed by gel filtration of Sephadex G-25 (Pharmacia Fine Chemicals, Sweden) column chromatography, and then DEAE (diethylaminoethyl)-cellulose ion exchange chromatography (Nacalai Tesque, Inc., Kyoto). The F/P (1:2) ratio was determined by measuring the absorbance at 280 and 495 nm. This FITC-labeled MAb was used for direct IF test.

Serological analyses. For the NT test, about 200 PFU/ml of virus was incubated for 60 min at 37 C with fourfold serial dilutions of MAbs in MEM-CS. The virus-antibody sample was adsorbed to RK13 or HeLa cells for 60 min at 37 C. After 48 to 64 hr of incubation at 37 C, plaques were visualized by staining with 0.13% crystal violet in 2% ethanol. The titer of the antibodies was determined as the reciprocal of the highest fourfold dilution neutralizing 50% of the PFU. The IP assay has been described in detail elsewhere (11). The WB techniques with MAbs by the method of Towbin et al. (26) with slight modifications have been described in detail elsewhere (11).

Results

Establishment and Characterization of MAbs

Seven MAbs were obtained from mice immunized with the CPV (LB red strain)- or the VV (Ikeda or Lister strain)-infected cells. The specificities and the cross-reactivities of these antibodies with cells infected with various strains of CPV, VV, EV and SFV were examined by IF, IP, WB and several biological and serological analyses. The antibodies reacting with the known major antigens, i.e., the common antigen of all poxviruses, probably involved the NP and NT antigen.

Table 1. Reactivity of monoclonal antibodies (MAbs) with various strains of orthopoxviruses

Group	MAbs	Immunogen	Isotype	IP (MW)	WB (MW)	NT (titer)	Cross-reactivity in IF (IF pattern)											
							CPV[c]					EV		VV				SFV
							LB[d]	Am	53	58	60	IS	HA	IK	LI	IW	IJ	OA
A	C1321	LB red	G1	65 K[a]	65 K[a]	<16[b]	G[e]	G	G	G	G	G	G	G	G	G	G	P
	C8911	LB red	G1	65 K	65 K	<16	G	G	G	G	G	G	G	G	G	G	G	P
	V1141	Ikeda	G3	65 K	65 K	<16	G	G	G	G	G	G	G	G	G	G	G	P
B	C4413	LB red	G1	40 K	40, 65 K	<16	P	P	P	P	P	P	P	P	P	P	P	(P)[f]
C	C3716	LB red	G1	NT[g]	28 K	4,096	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	−
	V1771	Ikeda	G2a	28 K	28 K	1,024	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	−
	VL101	Lister	G1	NT	28 K	4,096	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	SD	−
	ATI	LB red	G1	160 K	160 K	<16	ATI	ATI	−[h]	−	−	−	−	−	−	−	−	−

[a] Molecular weight ($\times 10^3$) of polypeptide upon immunoprecipitation (IP) or immunoblotting (WB).

[b] Neutralization (NT) titers, expressed as the reciprocal of the highest fourfold dilutions of antibodies, giving a positive reaction.

[c] Cross-reactivities with cowpox virus (CPV), ectromelia virus (EV), vaccinia virus (VV) and Shope fibroma virus (SFV) in the immunofluorescence (IF) test.

[d] LB, LB red strain; Am, Amsterdam strain; 53, 53 strain; 58, 58 strain; 60, 60 strain, IS, Ishibashi strain; HA, Hampstead strain; IK, Ikeda strain; LI, Lister strain; IW, IHD-W strain; IJ, IHD-J strain; OA, OA strain.

[e] Staining patterns in the IF test: G, granular staining; P, pin spot-like staining; SD, surface and diffuse staining; ATI, A-type inclusion body staining pattern.

[f] The antibody gave faint positive reactions in the IF test.

[g] NT, not tested.

[h] −, negative reaction.

Seven MAbs were characterized and classified into three groups according to their IF, IP and WB analyses. Results of the reactivities of the seven MAbs directed against common antigens are summarized in Table 1 and are described below in detail.

Three MAbs (C1321, C8911 and V1141) in the group A reacted with the CPV strains (LB red, Amsterdam, 53, 58 and 60), EV strains (Ishibashi and Hampstead) and VV strains (Ikeda, Lister, IHD-W and IHD-J). MAbs C1321 and C8911 clones were derived from mice immunized with the LB red strain of CPV, and the MAb V1141 clone was obtained from mice immunized with the Ikeda strain of VV. MAbs C1321 and C8911 were isotype IgG1, and MAb V1141 was isotype IgG3. All of these MAbs reacted with the strains of CPV, EV and VV by the IF test (Table 1), and with granular patterns at the cytoplasm of infected cells (Fig. 1a). These MAbs reacted with the strains of CPV, EV and VV by IP and WB (Table 1), and with a polypeptide band of molecular weight 65,000 (65 K) in the infected cells (Fig. 2). These MAbs also reacted with the SFV of the genus *Leporipoxviridae* (OA strain) in the IF test. These antibodies gave negative reactions in the NT and MIF tests, and with MCV-, measles virus (Edmonston strain)-, herpes simplex virus (type 1)- and mock-infected cells.

On the other hand, the C4413 clone (group B) reacted with a 40 K polypeptide on IP and with two polypeptides of 65 K and 40 K on WB (Fig. 2), and gave a pin spot-like cytoplasmic fluorescent staining pattern (Fig. 1b). The cross-reactivity of C4413 with each virus and with the 65 K polypeptide was the same as those of group A. However, the IF staining and polypeptide patterns of MAb C4413 appeared to be different from these of the clones in group A.

The C3716, V1771 and VL101 MAbs had neutralizing activity and each NT titer was 1,024 to 4,096 (Table 1). The C3716 clone was established from mice immunized with the LB red strain of CPV, and the V1141 and V1771 clones were established from mice immunized with the Ikeda strain and Lister strain of VV, respectively. These MAbs reacted with 28 K polypeptides on IP and on WB (Fig. 2). These antibodies gave positive reactions with cells infected with CPV, EV or VV, but not with SFV in the IF and WB. The reaction was positive in the MIF test. These antibodies gave negative reactions with MCV-, measles virus-, herpes simplex virus (type 1)- and mock-infected cells. The IF staining by these antibodies was surface and cytoplasmic diffuse (Fig. 1c).

Possibility of Rapid Diagnosis Using MAbs

The direct IF test using the FITC-labeled MAbs shortens the time needed for the procedures. Two types of MAbs, V1141 (anti-NP) and VL101 (anti-NT) were chosen for development of the serological detection

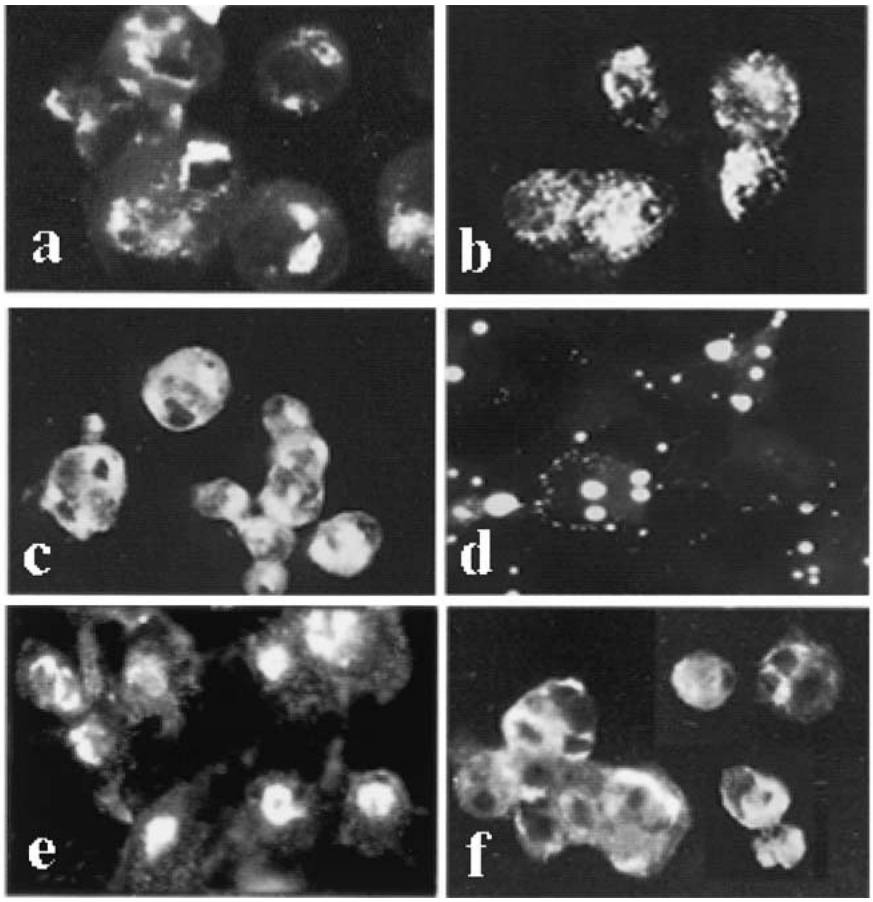

Fig. 1. Characteristic appearance of indirect (a–d) and direct (e, f) immunofluorescence (IF) test after staining of CPV (cowpox virus)-infected RK13 cells with each monoclonal antibody (MAb). The cells infected with CPV (LB red strain) were smeared and fixed with cold acetone for 5 min. For indirect IF test (a–d), MAb was added as the first antibody and incubated for 1 hr at 37 C. After washing with PBS, FITC-conjugated goat anti-mouse IgG (Cappel Lab., Westchester, Penn., U.S.A.) as the second antibody was reacted for 30 min at 37 C. For the direct IF test (e, f), FITC-conjugated MAb was dropped and incubated for 30 min at 37 C. MAbs: (a) C1321, granular fluorescence of cytoplasm; (b) C4413, pin spot-like staining in the cytoplasm; (c) C3716, cell surface and diffuse cytoplasmic staining; (d) ATI, staining of cytoplasmic A-type inclusions as a control; (e) FITC-labeled V1141, granular fluorescence of cytoplasm; (f) FITC-labeled VL101, cell surface and diffuse cytoplasmic staining.

methods for the direct IF test. The smear of infected cells was fixed with cold acetone for 5 min. The FITC-labeled MAb was dropped on the smear and incubated for 30 min at 37 C. The smear was then washed with PBS (phosphate buffer saline, pH 7.0) and mounted with glycerin for fluorescence microscopic examination. This procedure takes only 45 min. The FITC-labeled V1141 showed granular patterns at the cytoplasm of infected cells (Fig. 1e), and cross-reacted with cells infected with CPV, VV, EV and SFV. The labeled VL101 showed a surface diffuse pattern at the cytoplasm of infected cells (Fig. 1f), and cross-reacted with cells infected with CPV, VV and EV.

Discussion

Three MAbs (C1321, C8911 and V1141) in group A recognized a common antigen in all the poxviruses (probably NP antigen, although this has not been absolutely established), since they both reacted with the 65 K polypeptide (6, 11) and cross-reacted with all the poxvirus-infected cells (29). We also found that the 65 K polypeptide was detected by the WB of CPV virions purified from infected cells (data not shown). Wilton et al. (28) reported several MAbs against VV which recognized the core complex components and reacted with cytoplasmic inclusions (called B-type inclusions) widely ranging in size. The possibility is that the NP proteins are highly immunogenic antigens. The NP antigen is associated with the B-type inclusions, and not with ATI (9). On the other hand, the C4413 clone (group B) reacted with a 40 K polypeptide on IP and with two polypeptides of 65 K and 40 K on WB. This was examined by peptide mapping experiments involving

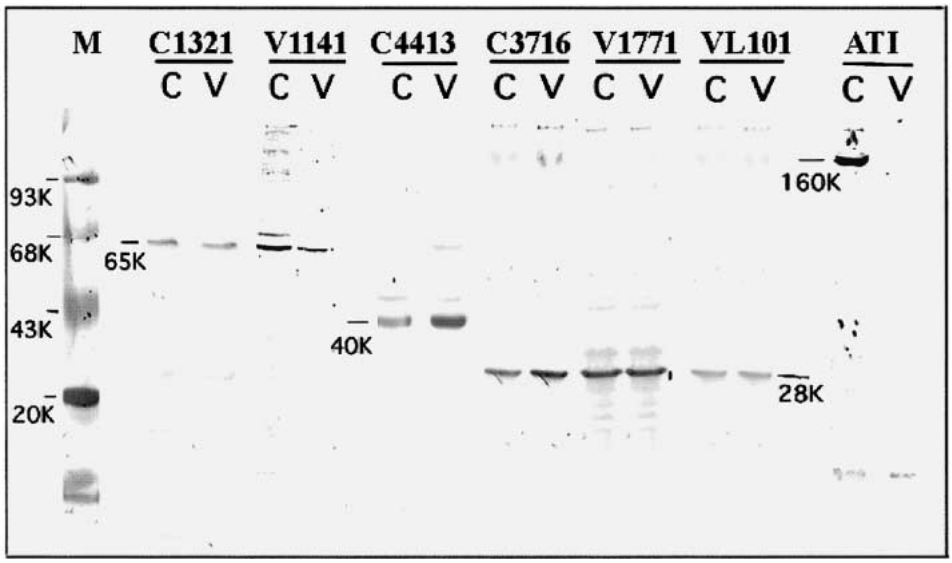

Fig. 2. Western blot (WB) of polypeptides from CPV (cowpox virus)- and VV (vaccinia virus)-infected RK13 cells reacting with monoclonal antibodies (MAbs). The results shown are for MAbs, C1321, V1141, C4413, C3716, V1771, VL101 and ATI (as a control), and for antigen, LB red strain of CPV (C) and Lister strain of VV (V). Proteins in the cells were boiled for 2 min, lysed with sample buffer (about 10 to 20 μg/well) and SDS-PAGE was conducted in 10% polyacrylamide gels. The separated proteins were transferred to nitrocellulose sheets which were incubated overnight at room temperature with the indicated MAbs (at 1:1,000 dilution of ascites), and then incubated with horseradish peroxidase (HRPO) conjugated goat anti-mouse IgG (Cappel Lab.) for 1 hr at 37 C and treated with substrate (4-chloro-1-naphthol). Calibration kits were used for the molecular weight determination (M); numbers on the left side indicate the apparent molecular weight (×1,000). Each MAb reacted with all strains of the genus *Orthopoxvirus*, with the major polypeptide with an apparent molecular weight of 65 K, 40 K or 28 K.

the partial proteolysis of these 65 K polypeptides by V8 protease digestion (1). The peptide pattern of the 65 K polypeptide reacting with C4413 was different from that recognized by clones of group A. The cross-reactivity of C4413 with each virus and with the 65 K polypeptide was the same as those of group A. However, the IF staining and polypeptide patterns of MAb C4413 appeared to be different from these of the clones in group A.

The C3716, V1771 and VL101 MAbs had neutralizing activity. These MAbs recognizing an envelope protein of VV have been reported to show neutralizing activity and to react with the 14 K polypeptide (27) or the 28–29 K polypeptide (14, 20). The C3716 clone was established from mice immunized with the LB red strain of CPV, and the V1141 and V1771 clones were established from mice immunized with the Ikeda strain and Lister strain of VV, respectively. These antibodies cross-reacted with cells infected with each strain of CPV and VV. Therefore, the 28 K polypeptide associated with neutralization shows a high degree of protein conservation in the *Orthopoxvirus* genus.

The final aim of this study was to establish a diagnosis of the smallpox infection. The IF test using MAbs was examined for the detection of the orthopoxviruses. Although the methods using PCR and EM were already available for detecting poxvirus, the IF test using MAbs might be no less specific than PCR and EM. Also, the IF test is simpler, manageable at a lower cost and applicable for more samples than the PCR and EM diagnoses. In the indirect IF test, MAbs will be used as the primary antibodies for the detection of the poxvirus antigen in fresh tissue impression smears. This assay required only 2–3 hr to detect the viral antigen. The ELISA (enzyme-linked immunosorbent assay), immunochromatography and microarray assays using MAbs might also be rapid and capable ways of detecting the virus in tissue samples. In preliminary study, ELISA using our MAbs will make it possible to detect the orthopoxviruses. However, a treated or solubilized antigen sample has to be prepared. In contrast, in the IF test, only fresh tissue impression smears from skin lesions will serve this purpose. In our preliminary study, we determine that the direct IF test will prove to be sensitive and reliable against infected tissues of laboratory animal. BALB/c mice were infected intramuscularly or intracutaneously with Lister strains of VV. Three or 4 days later, tissue impression smear samples were obtained from vesicular or pustular lesions and examined with the direct IF test. The MAbs NT1 and NP1 showed clear positive reactions as well as tissue culture infected cells. It is important to prepare for the

rapid diagnosis of smallpox in an emergency. The direct IF test requires only 45 min to perform and multiple samples can be tested at one time.

Relationships between poxviruses were evaluated by comparing the genome size, number of unique genes, gene arrangement and phylogenetic analyses of protein sequences (3). Genus *Avipoxvirus* is the most divergent. The next most divergent is *Molluscipoxvirus*, whose sole member, MCV, infects only man. Within the genus *Orthopoxvirus*, monkeypox virus, EV, CPV and VV are closely related to variola virus and are distinct from SFV of the genus *Leporipoxvirus*. Immunofluorescence staining technique using polyclonal antibodies has been examined (15, 25). The results indicated high sensitivity for the IF test in diagnosing variola major, but also showed a high rate of false-positive results (25). Our previous study, hyper immune mouse sera to VV showed faint positive reactions with MCV, which has low homology to orthopoxviruses. Our MAbs did not react in the least with MCV. These results suggest that MAbs are more specific for orthopoxviruses.

It has been reported that MAbs reactive against each of the five outer layer proteins neutralized infectivity (20). Several neutralization epitopes were also mapped to within the short consensus repeat domain (18). Further studies are necessary to define the molecular location of these epitopes recognized with our MAbs, and to examine the reaction to synthetic peptides homologous to variola virus. Although we actually cannot attempt to develop human samples, it is also necessary to demonstrate that these MAbs react with infected cells with monkeypox virus belonging to the same genus. It is important to take measures to deal with the situation and to prepare for the diagnosis of smallpox in an emergency. The direct IF test using MAbs is a rapid, simple and useful routine screening for multiple samples of tissue impression smears from skin lesions in most laboratories or institutes. These are the major advantages of the assay developed in this study. Virus isolation, PCR or EM examination could be used as a definite diagnosis for smallpox infection.

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