Elsevier has created a Monkeypox Information Center in response to the declared public health emergency of international concern, with free information in English on the monkeypox virus. The Monkeypox Information Center is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its monkeypox related research that is available on the Monkeypox Information Center - including this research content - immediately available in publicly funded repositories, with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the Monkeypox Information Center remains active.

VIROLOGY 163, 133–144 (1988)

Epitope Mosaic on the Surface Proteins of Orthopoxviruses

YASUO ICHIHASHI[1] AND MASAYASU OIE

Department of Virology, Faculty of Medicine, Niigata University, Asahimachi, Niigata 951, Japan

Received July 24, 1987; accepted November 4, 1987

Epitopes on the surface components of orthopoxviruses were analyzed with monoclonal antibodies (MAbs) against monkeypox and vaccinia viruses by enzyme-linked immunosorbent assay (ELISA), Western blotting (WB), radioimmunoprecipitation (RIP), and competitive binding inhibition assay (CBIA). When compared by ELISA, three vaccinia virus strains exhibited a similar reactivity to 99 tested MAbs despite their remote passage history. All five isolates of monkeypox virus closely resembled one another, irrespective of the host species (human, monkey, squirrel) from which they were isolated. Taterapox virus reacted similar to vaccinia virus against 97 of the 99 tested MAbs, and reacted with 2 MAbs which were cross-reactive with monkeypox and mousepox. Mousepox and cowpox viruses reacted with these MAbs in a species-specific manner: MAbs reactive to cowpox virus distinctly differ from those reactive to mousepox virus. Of the 99 tested MAbs, 32 reacted with all the 11 tested orthopoxviruses, indicating that the corresponding epitopes existed in all the viruses. Fifty-four MAbs reacted with two or more virus species and were classified as partially common MAbs. Eight MAbs were apparently type-specific for monkeypox, and five were specific for vaccinia and taterapox viruses. No strain-specific epitope was detected. Sera of monkeypox-infected patients, when analyzed by CBIA, interfered with the binding of monkeypox-specific MAb H12C1 but not of vaccinia-specific MAb G6C6. Sera of monkeypox-infected patients who had been vaccinated competed against both MAbs, demonstrating the original antigenic sin phenomenon. The two MAbs could distinguish between the sera of monkeypox patients and those of vaccinated persons. However, the serum of a smallpox patient was competitive against these apparently vaccinia- or monkeypox-specific MAbs. Three of the eight monkeypox-specific epitopes were recognized by the above CBIA test, which suggests that they also exist in smallpox virus. The mosaic-like combination of common epitopes and the small number of type-specific epitopes manifested the antigenic characteristics of orthopox viruses. The species boundary was obscured due to the partially common epitopes, but the total composition of epitopes was stable enough to maintain the antigenic species-specificity. The mutual relationship of the orthopoxviruses was visualized in a three-dimensional network.

INTRODUCTION

Reactivity with specific antibody is a reliable and the most convenient measure for the taxonomy of many viruses. Several serological methods have been employed, among which the neutralization test is the definitive criterion for classification. However, orthopoxviruses are currently identified based on the total of their biological characteristics, including genome restriction enzyme maps (Baxby, 1975; Muller *et al.*, 1978; Dumbell and Archard, 1980; Wittek, 1982; Nakano, 1985; Esposito and Knight, 1985; Fenner, 1976, 1985), although they are closely related in all serological tests employed so far (Cho and Wenner, 1973). To clarify the nature of species among orthopoxviruses, one has to explain the reason for such high cross-reactivity among the members of a group of viruses that have such distinct individual characteristics.

Recently we produced a number of hybridoma cell lines that secrete monoclonal antibodies (MAbs) against monkeypox and vaccinia viruses (Oie and Ichihashi, 1987). To characterize the specificities of epitopes on surface components of several orthopoxviruses, we used these MAbs and found that each orthopoxvirus has a specific composition of common epitopes. Orthopoxviruses seem to express immunological species-specificity as a total configuration of the epitopes, which suggests recombination as the mechanism that generates the mosaic-like epitope composition.

[1] To whom requests for reprints should be addressed.

MATERIALS AND METHODS

Virus and cells

Vaccinia virus (strains IHD-J, WR, and Elstree), taterapox virus (Lourie *et al.*, 1975), and monkeypox virus (strains Copenhagen, Zaire 599, CDC 21, CDC 218, and CDC 240) were propagated in KB cells as described previously (Ichihashi and Oie, 1982). The CPR-C1 strain of cowpox virus and the Ishibashi strain of mousepox virus were grown in Vero cells. The genetic markers of some of these viruses have been described (Ichihashi and Matsumoto, 1969; Mackett and Archard, 1979); however, the relationships among

0042-6822/88 $3.00

monkeypox isolates have not been fully studied. All the isolates have been tentatively referred to as strains in this paper. Each virus was purified by sucrose density gradient centrifugation (Joklik, 1962) and titrated in Vero cells (Oie, 1985).

Production and selection of MAbs

Inbred BALB/c mice were immunized with purified Zaire 599 monkeypox or IHD-J strain vaccinia virus (0.1 mg of uv-inactivated monkeypox virus or 10^7 PFU of vaccinia virus) injected twice intraperitoneally 4 weeks apart. The mice were given booster injections 2 weeks after the second injection. Three days after the booster injection, the splenocytes were fused with P3-X63-Ag8.653 myeloma cells (Kohler and Milstein, 1976). Hybridoma cells secreting specific antibodies were identified by enzyme-linked immunosorbent assay (ELISA). The monkeypox or vaccinia antigen for the first screening was a homogenate of KB cells infected with monkeypox or vaccinia virus. Each positive clone was reexamined by the ELISA test, in which the test antigen was a purified virus. The monkeypox- or vaccinia-specific MAbs were examined further by Western blotting (WB) and then by radioimmunoprecipitation (RIP). The monoclonality of MAbs was checked by formation of a single L-chain band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) profiles, and if necessary the hybridoma cells were selected by single-cell culture.

Radioimmunoprecipitation

KB cells were infected with Zaire 599 strain monkeypox virus. Medium containing [^{35}S]methionine (New England Nuclear, 10 μCi/ml, in methionine-free RPMI 1640 medium supplemented with 2% dialyzed fetal calf serum) was added to the cultures at 9 hr after infection. The labeled cells were harvested at 24 hr after infection, homogenized in 1 m*M* Tris buffer, pH 7.5, and centrifuged at 2000 rpm for 10 min. The supernatant fraction was centrifuged onto a 30% sucrose cushion at 18,000 rpm for 40 min. The crude virus fraction was dissociated by addition of lysis buffer (0.2% SDS, 0.5% deoxycholate, 0.5% Nonidet-P40, 1 m*M* phenylmethysulfonyl fluoride, 50 m*M* Tris–HCl, pH 7.5). The lysate was sonicated for 2 min and centrifuged at 100,000 *g* for 40 min. The supernatant and an equal volume (100 μl) of culture fluid of hybridoma cells were mixed and kept at 4° for 16 hr. Affinity-purified anti-mouse IgG (Bio-Rad, diluted 1:3000, 100 μl) was added to the mixture which was then incubated at 37° for 1 hr. The immune complex was recovered by adsorption to formalin-fixed Staphylococcus aureus Cowan I (Cullen and Schwartz 1976).

Electrophoresis

SDS–PAGE and WB were performed as previously described with a slight modification (Ichihashi *et al.*, 1984; Ichihashi, 1981; Towbin *et al.*, 1979). Skim milk solution (0.5% skim milk, 100 m*M* NaCl, 50 m*M* Tris–HCl, pH 8.0) instead of bovine serum albumin solution was used as blocking and washing solutions in the WB.

Competitive binding inhibition assay (CBIA)

CBIA was performed following the method of Voller *et al.* (1976) with a slight modification. In brief, purified monkeypox or vaccinia virus was suspended in carbonate buffer (pH 9.6) at a concentration of 2 μg/ml of virus. 100-μl volume of virus suspension was added to each well of an ELISA plate (Falcon Pro Bind plate) and left for 16 hr at 4°. After adsorption, the supernatant containing the unadsorbed virus was discarded and replaced by 100 μl/well of blocking solution (50 m*M* Tris buffer, pH 8.0, 100 mM NaCl, 0.5% skim milk). The plates were incubated at 37° for 1 hr. Reference sera were anti-monkeypox virus monkey serum (courtesy of Dr. T. Kitamura, NIH, Tokyo), anti-vaccinia virus rabbit serum (supplied by Dr. J. H. Nakano, CDC, Atlanta), and sera of 1- to 10-year-old children (negative control sera). Each serum was diluted serially with blocking solution, and 50 μl was added to each well. The monoclonal antibody solutions (monkeypox H12C1 MAb or vaccinia G6C6 MAb) were diluted with blocking solution. The concentration of the MAb was determined by preliminary test to yield OD_{492} = 2 against a given amount of antigen. Fifty microliters of the MAb solution was added to each well. The plates were incubated for 2 hr at 37° and washed three times with blocking solution for 5 min. An anti-mouse IgG peroxidase conjugate (Bio-Rad, diluted 1:3000 with blocking solution, 100 μl) was added to each well. The plates were incubated at 37° for 1 hr and washed three times with blocking solution. Fifty microliters of substrate–dye solution (*o*-phenylenediamine 0.4 mg/ml, H_2O_2 0.006%, citrate–phosphate buffer, pH 5.0) was added to each well. The reaction was terminated by adding 50 μl of 2 *M* sulfuric acid. The intensity of the reaction was measured with a Titertek EIA reader at wavelength of 492 nm. A computer program written by Mr. Kogawara was used to calculate the competitive antigen binding inhibition rate and to plot the best fitting line.

RESULTS

Selection of monoclonal antibodies specific for monkeypox or vaccinia virus

Anti-monkeypox virus hybridoma cultures (7 × 1200 wells) were tested by ELISA, and the results showed

that 155 wells produced MAbs reactive with monkeypox but not with vaccinia virus antigens. In 1200 wells of anti-vaccinia virus hybridoma cultures, 28 cultures produced MAbs specifically reactive with vaccinia virus cultures, 28 cultures produced MAbs specifically reactive with vaccinia virus antigens. More than 600 hybridoma clones produced cross-reactive MAbs.

The specificity of the MAbs against viral proteins was tested by the WB test, and the molecular sizes of target proteins were determined by comparison with the sizes of vaccinia virus proteins (Oie and Ichihashi, 1981; Ichihashi *et al.*, 1984). Figure 1 shows the close similarity of the structural proteins of monkeypox, cowpox, and vaccinia viruses. There were differences in the sizes of several proteins, but no type-specific protein species were detected except for the lack of VP32K in the WR strain of vaccinia virus. The WR and IHD-W strains of vaccinia virus are VP32K$^-$ (Oie and Ichihashi, 1981).

The relationships of proteins, such as VP37K the size of which was different among orthopoxviruses, were determined by cross-reactive MAbs. To avoid

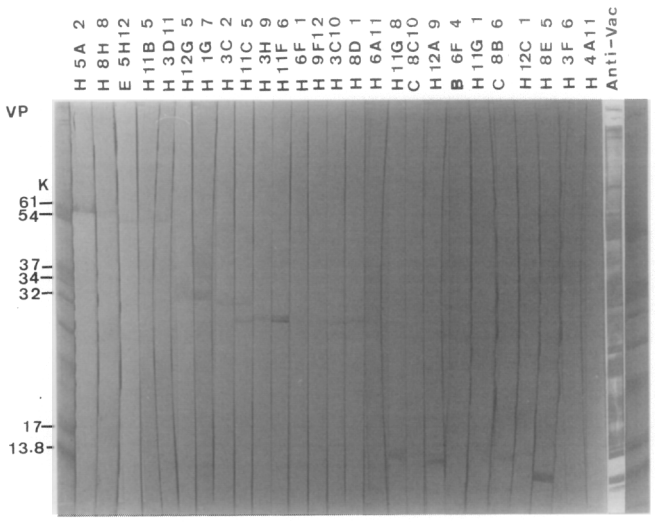

FIG. 2. WB profiles using anti-monkeypox MAbs. Purified Zaire 599 strain monkeypox virus was electrophoresed, blotted onto a nitrocellulose paper, and stained by the method described under Materials and Methods. Lane Anti-Vac was stained using anti-vaccinia hyperimmune serum; on the right-side lane are profiles of the blotted proteins stained with amido black.

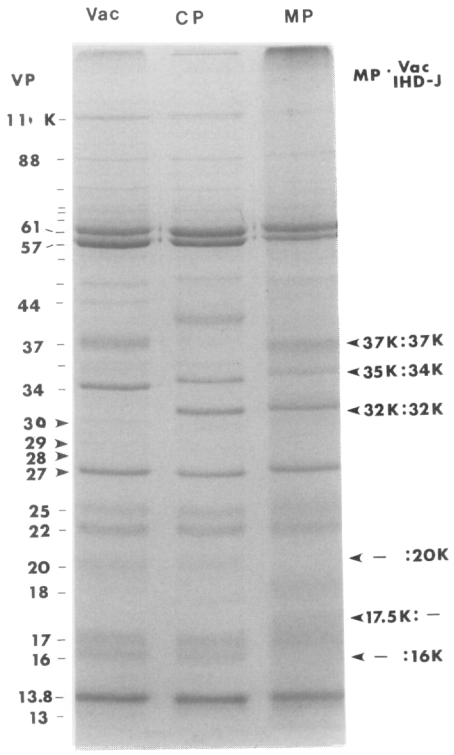

FIG. 1. SDS–PAGE profiles of WR strain vaccinia (Vac WR), Brighton strain cowpox (CP), and Copenhagen strain monkeypox (MP) viruses, 12.5% gel, Coomassie blue staining. Differences in the apparent sizes of viral proteins are indicated on the right. VP37K glycoprotein was similar in size in Vac and MP, but appeared at 41K position in CP. VP34K appeared at 35K position in MP. VP20K and VP16K were not found in MP. The 17.5K protein of MP was not detected in Vac.

confusion, we identified monkeypox virus proteins with similar vaccinia virus proteins. The vaccinia virus 17K protein corresponded to the previously reported monkeypox-specific 15.5K antigen (Roumillat *et al.*, 1984; Harper *et al.*, 1979).

MAbs specific against monkeypox antigen in the ELISA test were examined by WB and RIP tests. Seven proteins of monkeypox virus (VP61K, 54K, 34K, 32K, 17K, 13.8K, 13K) reacted with monkeypox-specific MAbs by the WB test (Fig. 2). These proteins were components of the virion outer membrane (Oie and Ichihashi, 1981). Since these MAbs were selected by the ELISA using purified virus as antigen, the epitopes of each protein were probably exposed on the surface of the virus outer layer. The seven proteins retained their specificity against monkeypox even after being separated from the virion. Several anti-monkeypox virus MAbs which reacted in monkeypox-specific manner in screening by ELISA were cross-reactive with similar-sized vaccinia virus proteins in the WB test, and vice versa. In the RIP test, VP57K basic protein adsorbed nonspecifically to the immunosorbent (*Staphylococcus aureus* Cowan I), and two single proteins and three protein complexes (VP54K–VP61K–VP88K, VP34K, VP22K–25′K, VP17K–VP20K–VP25K–VP54K, and VP13.8K) reacted with the MAbs (Fig. 3).

By WB, MAb H12C1 reacted with VP17K, indicating that a specific epitope was carried by VP17K, but by RIP, VP54K–VP20K–VP17K complex was precipitated. This complex suggests that a stepwise dissoci-

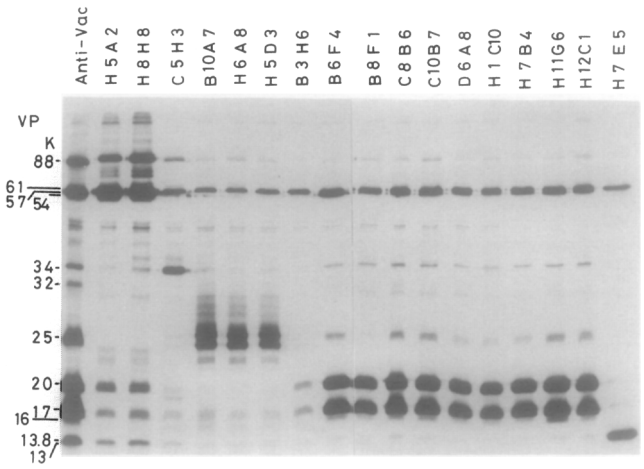

FIG. 3. RIP profiles of Zaire 599 strain monkeypox virus antigens precipitated by MAbs specific for monkeypox virus. The left-most lane is a reference lane in which anti-vaccinia hyperimmune serum was used.

ation of monkeypox virus had occurred when the virus was treated with detergents for testing with RIP.

The immunoprecipitate formed by MAb H12C1 was dissociated with nonreducing lysis buffer and electrophoresed in a column gel. The column gel was soaked in a lysis buffer containing adequate reducing agent, and was subjected to the second-dimension electrophoresis in a slab gel. Figure 4a shows that VP17K and VP25K migrated as a complex of a large molecular size in the first dimension, and separated into 25K and 17K proteins in the second-dimension electrophoresis. VP20K migrated in two molecular sizes of 30K and 20K in the first dimension and as 20K in the second dimension. When a duplicate sample was electrophoresed under a reducing condition in both dimensions, VP20K appeared as one spot and VP25K–VP17K formed a rectangular arrangement (Fig. 4b). Since the results shown in Fig. 4a were obtained by using a nonreducing condition and those for Fig. 4b by using a reducing condition, it was concluded that VP25K of monkeypox virus was a disulfide-linked homodimer of VP17K and that the protein constituted a high-molecular-weight polymer complex under a nonreducing condition, a phenomenon the same as that found in vaccinia virus reported previously (Ichihashi, 1981). VP30K is a homodimer of VP20K, but its disulfide linkage was much weaker than that of the VP25K–VP17K complex unit. The tailed profile of VP54K (Fig. 4b) may be due to imcomplete dissociation of VP16K from the complex. VP54K and VP16K were probably forming a heterocomplex. VP54K, VP34K, and VP13.8K appeared on the diagonal line (Fig. 4b), indicating that VP34K and VP13.8K have an affinity for VP20K–VP17K or VP54K–VP16K complexes when the complex is maintained by the disulfide linkage.

From the identification of the protein composition of the immunoprecipitate produced by MAb H12C1, it appeared that a fragment of the outer coat of the virus was trapped by the MAb specific for VP17K. Similarly, MAb H8H8, which is specific for VP61K, coprecipitated VP88K. MAb B10A7 specific for VP22K coprecipitated 25′K–VP22K complex. The 25′K protein is not well characterized. It may be another molecular species of 25 kDa because it did not show any VP25K feature (homodimer of VP17K).

A specific dissociation of virus particles by a lysis buffer for RIP produced the protein aggregates (complex) in the immunoprecipitates. The detergents in the

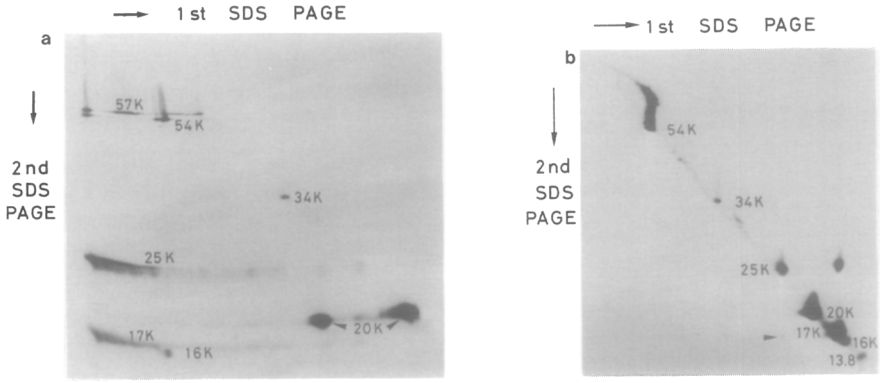

FIG. 4. Two-dimensional electrophoresis of an immunoprecipitate by MAb H12C1. Zaire 599 strain monkeypox virus was dissociated by lysis buffer for RIP and reacted with MAb H12C1. (a) The immunoprecipitate was dissolved in a nonreducing lysis buffer (2.3% SDS, 10% glycerol, 50 m*M* Tris–HCl, pH 6.5) and electrophoresed in a 12.5% gel column. The gel column was soaked in a reducing lysis buffer (2.3% SDS, 5% 2-mercaptoethanol, 10% glycerol, 50 m*M* Tris–HCl buffer, pH 6.5) for 1 hr, and then electrophoresed in a 12.5% gel slab. (b) A duplicate sample was processed in the reducing lysis buffer at the first electrophoresis. The second-dimension electrophoresis was done by the same procedure as in (a). Autoradiogram.

lysis buffer used for the RIP dissociated the virus outer layer at the detergent-sensitive sites, but could not sufficiently dissociate the virus to independent proteins. Increasing the amount of SDS for greater dissociation or adding 2-mercaptoethanol to the lysis buffer resulted only in the loss of antigenicity against some MAbs. The discrepancy between the results obtained by the WB and those obtained by the RIP may be because some epitopes were intact in the protein complex, but were denatured when the proteins were solubilized as a single protein. A further explanation could be that the outer coat layer of monkeypox virus is composed of disulfide-linked unit complexes (VP88K–VP61K–VP57K, VP54K–VP16K, 25K–VP22K, VP20K–VP17K) and inserted proteins (VP54K, VP34K, VP32K, VP13.8K, VP13K).

Members of MAb groups that reacted with the same viral protein in the same manner in RIP and WB tests may recognize the same epitope. MAbs (anti-VP61K, H5A2, H8H8 and anti-VP17K, B6F4, C8B6, H11G6, H12C1) were examined by the CBIA test, where one of the MAbs to be compared was labeled with ^{125}I and used as the probe. MAb H5A2 did not compete with H8H8. B6F4, C8B6, H11G6, and H12C1 did not compete with one another (data not shown). Each member of these MAb groups recognized different epitopes on the same protein.

Epitope composition of orthopox viruses

The type-specificity of MAbs was examined with respect to the following orthopoxviruses: monkeypox virus (strains Zaire 599, CDC 21, CDC 240, and Copenhagen), vaccinia virus (strains IHD-J, WR and Elstree), cowpox virus strain CPR C-1, mousepox virus strain Ishibashi-111, taterapox virus, and a monkeypox virus recently isolated from a squirrel in Zaire (strain CDC 218, Khodakevich *et al.,* 1986). Table 1 shows the reaction of 99 monkeypox and vaccinia MAbs against purified orthopoxviruses as examined by the ELISA.

Despite a remote passage history (Esposito and Knight, 1985), vaccinia virus strains possess very similar epitope compositions. Significant differences among the strains were found in the reactions against H1G7, H9F12, H1C10, and H5A1; however, all of these MAbs were produced by hybridomas raised from spleen cells of monkeypox-immunized mice, and reacted strongly with monkeypox viruses. Accordingly, the differences detected by these MAbs were not due to strain-specific epitopes, but indicated that the epitopes common to monkeypox and vaccinia viruses did not exist in specific vaccinia strains. In MAbs raised against vaccinia virus, G9F8, G1A10, G8D9, G6E2, and G3C1 did not react with mousepox, cowpox, and monkeypox viruses, but all of them were cross-reactive with taterapox virus.

Taterapox virus was isolated from a wild gerbil in Dahomey, Africa, at the time of an epidemic of human smallpox. The biological characteristics of the virus resembled those of variola minor virus (Lourie *et al.,* 1975), and the restriction enzyme map showed that the virus was a separate orthopoxvirus species (Esposito and Knight, 1985). However, the epitope composition of taterapox virus closely resembled that of vaccinia viruses. Out of the tested 99 MAbs, only 2 (H12D1 and I3A8) reacted differently between the two viruses. The 2 MAbs did not react with vaccinia but with taterapox, mousepox, and monkeypox viruses. Thus, the species-specificity of taterapox virus, evidenced by restriction enzyme mapping did not agree with its epitope composition. The present results suggest that the virus is a wild vaccinia or a a recombinant between vaccinia and another poxvirus whose epitopes are common to monkeypox virus. If taterapox virus were not so unique as to be an independent species, the epitopes reactive to the MAbs (G9F8 to G3C1 in the list) could be classified vaccinia-specific.

The reactions of Ishibashi 111 strain mousepox and CPR-C1 strain cowpox viruses with these MAbs distinctly differ from each other and from those of vaccinia and monkeypox viruses. There are significant differences between MAb groups reactive to mousepox virus and those reactive to cowpox virus. Since all the MAbs were raised against vaccinia or monkeypox, the positive reactions represented common epitopes existing in the species. The partially common epitopes, therefore, expressed type-specificity by their cowpox- or mousepox-specific compositions.

Monkeypox virus isolated from squirrel (strain CDC 218) and those isolated from human and/or monkey (strains CDC 21, 240, Zaire 599, and Copenhagen) have almost the same epitope compositions despite their remote isolation sites, long intervals of the isolation times, and difference in the host species from which they were isolated. A difference was lack of reactivity of Zaire 599 strain virus against vaccinia MAbs (G6C6, G6G11, G11B5, G3F4, and G8E2). CDC 21, CDC 240, and Copenhagen strains can be classified to a single strain. Eight MAbs (H7B4, H5B12, B6F4, C8B6, D6A8, H8G8, H11G1, and H12C1) reacted specifically with monkeypox virus.

Thirty-two out of 99 tested MAbs reacted equally well with all the tested viruses, and 54 MAbs were cross-reactive but reacted weakly or did not react with specific virus. Eight monkeypox-specific MAbs and five possibly vaccinia-specific MAbs were found, but no MAb reacted as being strain-specific. A panoramic

TABLE 1

EPITOPE DISTRIBUTION IN 11 ORTHOPOXVIRUSES

MAb	Antigen VP MW	Antigen (Purified virus) Vaccinia J	E	W	Tat	I-111	C1	Monkeypox Z	21	218	240	Cop
G 9F 8		+	+	+	+-	-	-	-	-	-	-	-
G 1A10		+++	+++	+++	+++	-	-	-	-	-	-	-
G 8D 9		++	++	++	+	-	-	-	-	-	-	-
G 6E 2		++	+	++	+	-	-	-	-	-	-	-
G 3C 1		++	++	++	+	-	-	-	-	-	-	-
G 3B10*	54K	+++	+++	+++	++	-	-	-		-		+-
G 12F11		+	+	+	+	+-	-	-	-	-	-	-
G 4G 9		+++	+++	+++	+	+++	+-	-	-	-	-	-
G 11D11*	34K	+++	+++	+++	+++	+++	+++	-		-		+-
G 11C11		+++	+++	+++	+-	-	+++	-	-	-	-	-
G 1B 5		+++	+++	+++	+++	-	+++	-	-	-	-	-
G 12B 5		++	++	++	+	-	+-	-	-	-	-	-
G 6C 6		+++	+++	+++	+++	-	+-	-	+-	+-	+-	+-
G 6G11		++	++	+++	+	-	+-	-	+-	+-	+-	-
G 11B 5		+++	+++	+++	+++	-	+-	-	+-	+	+	+-
G 3F 7		+++	+++	+++	+++	-	-	-	+-	+-	+-	+-
G 8E 2		+++	+++	+++	+++	-	-	-	+-	+-	+-	+-
G 9D 9*		++	++	++	++	+++	++	+-		+-		+
G 7H 5*	29K	++	++	+++	++	+	++	+-		+		++
G 7B 7		+++	+++	+++	+++	-	-	+	++	++	++	+
G 10G 8*	54K	++	+-	++	+++	-	-	++		+++		++
G 4H 6*	54K	+++	+++	+++	+++	-	-	+++		+++		-
G 10B10*	54K	+++	+++	+++	+++	-	-	+++		+++		+
G 10F 4*	29K	++	++	++	++	-	++	++		+++		++
G 11C 9*	54K	+++	+++	+++	+++	-	+++	++		++		++
G 12G 6*		+++	+++	+++	+++	-	+++	++		+++		++
H 11D12		+++	+++	+++	+++	+	-	+++	+++	+++	+++	++
G 8H 2*	54K	+++	+++	+++	+++	+-	+-	+++		+++		+++
H 5B 5		+++	+++	+++	+++	+-	+-	+++	+++	+++	+++	+++
H 6F 1	32K	+++	+++	+++	+++	+-	+-	+++	+++	+++	+++	+++
H 4F 4		+++	+++	+++	+++	+	+-	+++	+++	+++	+++	+++
G 10H 5*	54K	++	++	++	++	+	+	+		+		+
G 5C 1*	32K	+++	+++	++	+++	+++	++	++		++		++
H 1E 6		+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++
G 12C 9*	54K	+++	+++	+++	+++	++	+	+++		+++		+++
H 2E 9		+++	+++	+++	+++	+++	++	+++	+++	+++	+++	+++
H 1A 7		+++	+++	+++	+++	++	+++	+++	+++	+++	+++	+++
G 1D11*	17,25K	++	+++	++	+++	+++	+++	++		+++		+++
G 10B 6*	29K	+++	+++	+++	+++	+++	+++	+++		+++		+++
H 1F 4		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 3B 3		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 3C10	32K	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 3H 9	32K	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 4C 3		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 4D 5	17K	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 4E 3		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 4G 3		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 6A 5		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 6B 1		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

MAb	Antigen VP MW	Antigen (Purified virus) Vaccinia J	E	W	Tat	I-111	C1	Monkeypox Z	21	218	240	Cop
H 6G 5		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 6H11		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 7C12		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 7E 3		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 8D 1	32K	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 8G 2		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 9C 7		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 9D 1		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 9H 4		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 9H 7		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 10D 8		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 10F 5		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 11C 5	32,34K	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 11F 6	32K	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 10D 5		+++	+++	+++	+++	+	++	+++	+++	+++	+++	++
H 8D 7		+++	+++	+++	+++	+	+	+++	+++	+++	+++	+++
H 12A 6		+++	+++	+++	+++	+	+	+++	+++	+++	+++	+++
H 12G 5	54K	+++	+++	+++	+++	+	+	+++	+++	+++	+++	+++
H 12H 3		+++	+++	+++	+++	+	+	+++	+++	+++	+++	+++
H 4A11	13.8K	+++	+++	+++	+++	-	+++	+++	+++	+++	+++	+++
H 2A 2		+++	+++	+++	+++	-	+	+++	+++	+++	+++	+++
H 11F 2		+++	+++	+++	+++	-	+	+++	+++	+++	+++	+++
C 10B 7	17K	+++	+++	+++	+++	-	-	+++	+++	+++	+++	++
H 3D10		+++	+++	+++	+++	-	-	+++	+++	+++	+++	+++
H 3F 6	17K	+++	+++	+++	+++	-	-	+++	+++	+++	+++	+
H 4H11		+++	+++	+++	+++	-	-	+++	+++	+++	+++	+++
H 11D 1		+++	+++	+++	+++	-	-	+++	+++	+++	+++	+++
H 11G 6		+++	+++	+++	+++	-	-	+++	+++	+++	+++	+++
H 1G 7	17,34K	++	+	-	+++	-	+++	+++	+++	+++	+++	+++
H 3C 2	34K	+	-	+++	+++	+++	+++	+++	+++	+++	+++	+++
H 9F12	17K	+	+-	-	+-	+	+	+++	+++	+++	+++	+++
H 11G 8	17K	+	+	+	++	+-	+	+	+	+	+	+
H 12A 9	17K	+-	+	+	+	+-	+-	+++	+++	+++	+++	+++
H 1C10	17K	-	++	++	++	-	-	+++	+++	+++	+++	+++
H 5A 2	61K	-	-	+-	+-	+	-	++	+++	+++	+++	++
H 12D 1		-	-	-	+	-	-	++	+++	+++	+++	++
I 3A 8		-	-	-	+++	+++	-	+++	+++	+++	+++	+++
H 6A11	32K	-	-	-	-	+-	+++	+++	+++	+++	+++	+++
H 3F 4		-	-	-	-	-	+++	+++	+++	+++	+++	+++
H 5B 3		-	-	-	-	-	++	++	++	++	++	++
H 6H11		-	-	-	-	-	+++	+++	+++	+++	+++	+++
H 12D 5		-	-	-	-	-	+++	+++	+++	+++	+++	+++
H 7B 4	54K	-	-	-	-	-	-	+	+	+	+	-
H 5B12	17K	-	-	-	-	-	-	+	++	++	++	+
B 6F 4	17K	-	-	-	-	-	-	+++	+++	+++	+++	+++
C 8B 6	17K	-	-	-	-	-	-	+++	+++	+++	+++	+++
D 6A 8		-	-	-	-	-	-	+++	+++	+++	+++	+++
H 8G 8	32K	-	-	-	-	-	-	+++	+++	+++	+++	++
H 11G 1	17K	-	-	-	-	-	-	++	++	++	++	++
H 12C 1	17K	-	-	-	-	-	-	+++	+++	+++	+++	+++

J: Vaccinia, IHD-J.
W: Vaccinia, WR.
E: Vaccinia, Elstree.
C1: Cowpox, CPR-C1.
I-111: Mousepox, Ishibashi-111.
Tat: Taterapox.
Z: Monkeypox, Zaire-599.
21: Monkeypox, CDC-V86-I-21, WHO-Z-4125, Case 329, 1985.12.10, Bumba zone, Bonbanga village.
218: Monkeypox, CDC-V85-218, WHO-249, Isolated from a squirrel (*Funisciurus anerythrus*), 1985.7.21, Bumba zone, Bodjogi village. Infected squirrel was captured about 3 weeks before and 250 m away from where Case 298 occurred.
240: Monkeypox, CDC-V85-240, WHO-Z-3824, Case 298, 1985.8.15, Bumba zone, Bodjogi village.
Cop: Monkeypox, Copenhagen.

−: $OD_{492} < 0.2$
±: 0.2–0.4
+: 0.4–0.8
++: 0.8–1.6
+++: >1.6

MAbs whose code names begin with G were prepared from mice immunized with vaccinia virus strain IHD-J.
MAbs whose code names begin with B, C, D, or H were prepared from mice immunized with monkeypox virus strain Zaire 599.
* The MAb neutralizes vaccinia virus strain IHD-J.

view of orthopoxvirus epitopes using these MAbs indicated that orthopoxviruses share common epitopes in a species-specific manner. Thus, the antigenic species-specificity of a orthopoxvirus was expressed by the species-specific epitopes and common epitopes which had species-specific composition. Homology of the epitope compositions among vaccinia virus strains as well as among monkeypox virus strains indicated that these mosaic-like compositions of epitopes were highly stable and had been maintained through a long-isolated passage history.

The partially common epitopes in the orthopoxviruses probably derived from a common ancestral virus. If the epitopes of the ancestral virus were sequentially delivered to each species during evolution, the higher the homology of epitope composition the closer the relationship of the viruses, and linear line-up would be seen in accordance to distance from the ancestral virus. The distribution mode of the common epitopes in the species of orthopoxviruses, however, looked apparently random. To visualize the relationships among these 11 viruses, an index expressing relative differences was calculated (Fig. 5). The cluster distribution of vaccinia virus strains and of monkeypox virus strains indicated close relationships among the strains of each species. Taterapox virus was located near a vaccinia cluster a little apart from the line between vaccinia and monkeypox clusters. If the taterapox virus were direct progeny of the recombinant between vaccinia and monkeypox viruses, it would locate on the line. The location of taterapox in the model suggests therefore, one of the parent virus may not be monkeypox virus, even if the virus were recombinant. Mousepox and cowpox viruses located well apart from each other and from vaccinia and monkeypox viruses. The relationships revealed by the 99 MAbs were in three-diemensional net work, not in rod-like linear distribution which would appear if the common epitopes were produced by selective deletion from epitopes of a progenitor virus.

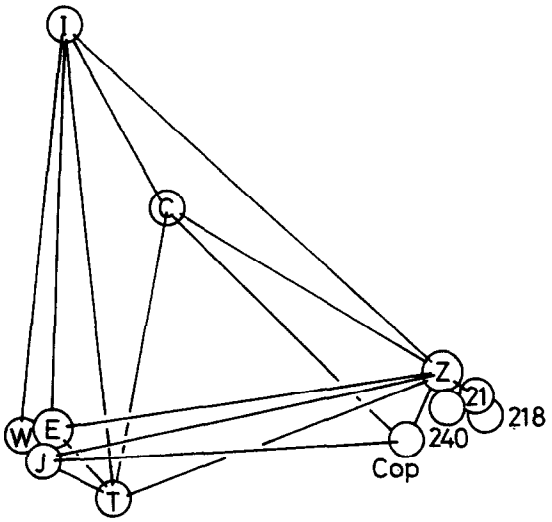

FIG. 5. Relative difference indexes of epitope compositions among orthopoxviruses visualized via a three-dimensional model. When there is a difference in reaction with a MAb between two strains, a value corresponding to the difference grades in the table below is subtracted from the total number of MAbs used in the comparison (99) and referred to as relative difference value (RDV) of the virus. The relative difference index between the two virus strains is calculated according to the formula

$$\text{Relative difference index} = \left(1 - \frac{\text{RDV for the virus}}{\text{Total number of MAbs}}\right) \times 100.$$

		Reaction of the virus to be compared				
		−	±	+	++	+++
Reaction of the reference virus to the MAb	−	0	−0.25	−0.5	−0.75	−1
	±	−0.25	0	−0.25	−0.5	−0.9
	+	−0.5	−0.25	0	−0.25	−0.5
	++	−0.75	−0.5	−0.25	0	−0.25
	+++	−1	−0.9	−0.5	−0.25	0

Competitive binding inhibition assay

During the screening of type-specific MAbs, we found that some MAbs were species-specific by the ELISA test, but cross-reactive in the WB or RIP test. In the ELISA test, purified virus was adsorbed to the surface of a plastic well and used as antigen. To prepare antigen for RIP, the virus was dissociated by detergents, and the virus was dissolved by detergents and a reducing agent in the WB test. When the antigens prepared for RIP or WB were tested by ELISA, some MAbs lost reactivity and some MAbs became cross-reactive. The difference in methods to produce the antigens in each test caused modification of epitopes and/or exposure of masked epitopes. On the other hand, a hyperimmune anti-vaccinia serum blocked reaction of any vaccinia MAbs in ELISA and WB tests. A pooled hyperimmune anti-vaccinia serum may contain antibodies against a wide range of vaccinia epitopes including usually masked epitopes. The specificity of a MAb was tested further by CBIA. If the pooled anti-vaccinia serum did not interfere with monkeypox MAb in the reaction with monkeypox virus, the target epitope of the MAb will be monkeypox-specific, otherwise common. Two MAbs were tested, namely, a MAb clone (monkeypox H12C1) that recognizes the monkeypox epitope on protein VP17K, and vaccinia G6C6 that lacks cross-reactivity with Zaire 599 strain monkeypox virus in the ELISA test. Polyclonal hyperimmune sera included for testing were anti-vaccinia virus rabbit sera, several sera of monkeypox patients (sup-

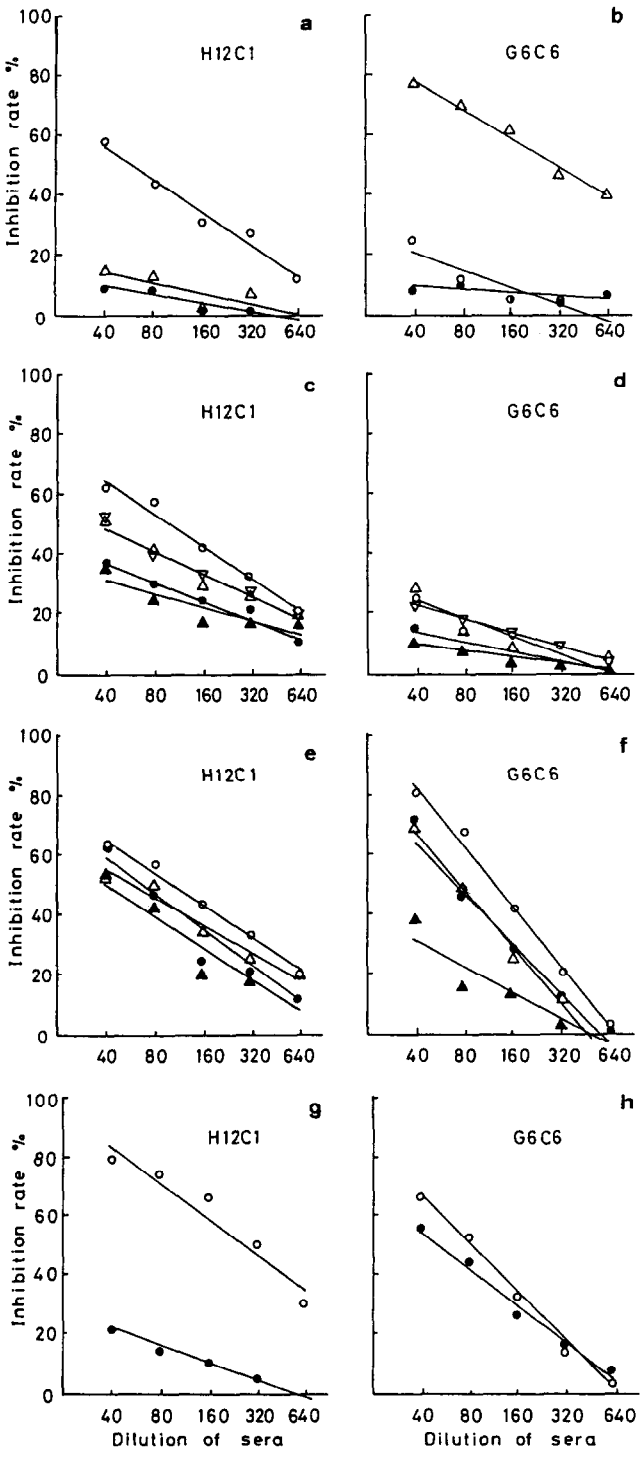

FIG. 6. CBIA of polyclonal antisera against MAbs specific for Zaire 599 strain monkeypox and IHD-J strain vaccinia virus. The ordinate indicates the inhibition rate (%) of the MAb binding to purified virus due to interference by the polyclonal serum. The abscissa indicates the dilution of the polyclonal antiserum. The polyclonal antisera are the same as those described in Table 2. (a and b) Anti-monkeypox monkey serum (○), anti-vaccinia rabbit serum (△), normal serum of children less than 10 years old (●). (c and d) Sera of monkeypox-infected patients who had not been vaccinated. S-97 (○), S-94 (△), S-113 (●), S-146 (▽), S-54 (▲). (e and f) Sera of monkeypox-infected patients who had been vaccinated. S-32 (○), S-182 (△), S-467 (●), S-307 (▲). (g and h) Serum from a smallpox-infected patient (○), anti-smallpox (Harvey strain) rabbit serum (●).

plied by Dr. J. H. Nakano), anti-monkeypox virus monkey sera, a serum of a smallpox patient (courtesy of Dr. T. Kitamura), and sera of Japanese children less than 10 years old (unvaccinated) and of adults more than 20 years old (vaccinated). The results are summarized in Table 2.

Figures 6a and 6b show that the polyclonal anti-monkeypox virus monkey serum competed with monkeypox MAb H12C1 for the epitope on monkeypox virus, but polyclonal anti-vaccinia virus rabbit serum and normal human serum did not compete significantly. The polyclonal anti-vaccinia virus rabbit serum reduced the amount of binding vaccinia MAb G6C6 to the epitope of vaccinia virus, but the polyclonal anti-monkeypox virus monkey serum did not. The inhibition rates of the sera from normal unvaccinated persons (Japanese children less than 10 years old) were less than 20% for both monkeypox H12C1 and for vaccinia G6C6 at a dilution of 1/40. These values represent a base line or background due to nonspecific inhibition under our experimental conditions. The inhibition rates of sera from vaccinated adults (Japanese more than 20 years old) were similar to those of unvaccinated children under 10 years old. Apparently, the anti-vaccinia virus titers in the vaccinated adults had decreased to background levels. The sera collected from persons in Zaire with no vaccination and no monkeypox illness showed low competitive binding inhibition activity against H12C1 and G6C6. Thus, the two MAbs were sufficiently type-specific. Therefore, using these MAbs as probe, we can determine which of the two polyclonal sera contained specific monkeypox or vaccinia antibody.

The inhibition rates of the sera from monkeypox patients ranged from 10 to 85% according to the time of serum sampling after onset of disease (Figs. 6c, 6d). One serum collected 1 day after the onset of disease showed an inhibition rate as low as that of the negative control sera (10%). Sera collected 9 days to 2 months after the onset of disease significantly inhibited the binding of MAb (>48%), but sera collected at 11 months or later showed a decreased rate of inhibition approaching the background level. The serum (S-97) of a monkeypox patient collected at 1 month after the onset of disease typically showed a high inhibition rate and a steep slope for the regression line against the anti-monkeypox MAb, and a lower inhibition rate and a

TABLE 2

CHARACTERISTICS OF VARIOUS SERA FROM MONKEYPOX AND SMALLPOX PATIENTS AND VARIOUS CONTROL SERA. COMPARISON OF ANTIBODY TITERS AS DETERMINED BY RIAA, CBIA, AND ELISA

Serum Numbers			Vaccination	Time	RIAA*	Inhibition rate** (%) H12C1	G6C6	CBIA	ELISA titer*** (x 10^{-3}) Anti-MP	Anti-Vac
CDC	Zaire	Case								
Sera from cases of monkeypox:										
V83-S-307	2751	126	-	2 M	M	50	30	M,V?	150	43
V83-S-397	2903	122	+	1 D		10	<5		11	5.2
V83-S-467	MF615	168	+	14 D	V	60	65	M,V	560	740
V84-S-32	MF772	190	+	10 D	V	65	82	M,V	1,000	720
V84-S-54	MF841	181	?	2 D	M?	30	12	M	90	72
V84-S-94	3229	208	-	2 1/2M	M	50	25	M	110	120
V84-S-97	3232	210	-	9 D	M?	67	25	M	500	300
V84-S-113	3248	212	-	11 M	M	38	12	M	24	11
V84-S-119	MF867	179	+	3Y8M	V?	36	12	M	21	40
V84-S-146	3267	196	+	1/2M	V?	48	22	M	190	390
V84-S-182	MF964	213	-	1 M	M	55	65	M,V	640	400
Sera from persons without monkeypox:										
V84-S-304	3543		-			<10	<5		1.5	1.8
V84-S-306	3546		+			22	<5		4.1	2.1
V85-S-49	3596		-			10	12		2.2	9.0
V85-S-53	3602		-			28	<5		0.64	1.0
WHO Kole surveillance 17766			-			22	<5		1.3	1.4
WHO Kole surveillance 17769			-			5	<5		0.47	0.42
WHO Kole surveillance 17773			-			12	<5		1.5	0.75
WHO Kole surveillance 17789			-			10	<5		1.4	0.94
Sera of children less than 10 years old, 20 samples			-		mean	<20 12	<20 10		0.30	0.32
Sera of vaccinated persons older than 20 years			+	10-15Y	mean	<30 12	<15 <5		2.1	2.3
Serum from a case of smallpox:										
IHS-73			+	25 D		85	74	M,V	440	840
Sera of immunized animals:										
Anti-vaccinia	CDC #4		-			15	77	V	20000	28000
Anti-monkeypox	Copenhagen		-			57	20	M	40	53
Anti-smallpox	VrIRS R7101		-			<5	70	V	710	630
Harvey	VrIRS R7102		-			25	40	V	750	420
	VrIRS R7103		-			25	56	V	1100	870
	VrIRS R7104		-			23	55	V	330	310
Monoclonal antibodies:										
Anti-vaccinia	G6C6								<0.001	30
Anti-monkeypox	H12C1								78	0.02

* Hutchinson *et al.*, 1977.

** Competitive binding inhibition assay (CBIA), measured at 1/40 dilution of the sample sera.

*** The ELISA titers of sample sera were assayed against purified monkeypox virus or vaccinia virus, and expressed with the dilution at which the reaction intensity was $OD_{492} = 0.5$.

lower slope for the regression line against the anti-vaccinia MAb.

Some sera of monkeypox patients showed similarly high inhibition rates against vaccinia and monkeypox MAbs, indicating that the sera contained antibodies against both vaccinia and monkeypox virus (Figs. 6e, 6f). Most of these patients had records of previous vaccination, therefore, the results indicated that the immunological memory of the epitope corresponding to the vaccinia-specific MAb was evoked by the second infection of antigenically closely related monkeypox virus, even though the later virus lacked the vaccinia-specific epitope. The increase of specific anti-vaccinia titer by superinfection with monkeypox virus is the phenomenon known as original antigenic sin or cross-stimulation. The question that remains to be resolved is the nature of the monkeypox virus component that triggers orthopoxvirus-specific helper T or memory B lymphocytes to recognize vaccinia-specific epitopes.

Type-specific epitopes that differentiate monkeypox and vaccinia viruses do exist, because MAbs such as H12C1 (against monkeypox virus) and G6C6 (against vaccinia virus) are distinguished between vaccinia and monkeypox viruses. CBIA was reliable for identifying the specific monkeypox antibody and the specific vac-

cinia antibody in the sera of individuals infected with monkeypox or vaccinia virus or both (Table 2).

Partially common epitopes in smallpox virus

A serum (IHS-73) collected from a smallpox patient who had been vaccinated showed high binding inhibition to both H12C1 and G6C6 MAbs. Concurrent assays indicated that the rabbit serum against the Harvey strain smallpox virus cross-reacted with the vaccinia-specific epitope, but not with the monkeypox-specific epitope (Figs. 6g, 6h). Accordingly, the Harvey strain smallpox virus shares the G6C6 epitope with vaccinia virus strain IHD-J, and the smallpox virus with which the smallpox patient had been infected shares the H12C1 reactive epitope with Zaire 599 strain monkeypox virus. There are two possible explanations for this observation: (a) The smallpox virus that infected the patient in Pakistan was actually a monkeypox virus; this is rather remote, because there is no evidence that monkeypox virus exists in the human population in Pakistan, or any other countries outside of Africa. (b) The epitopes existed in a partially common mode. The H12C1 epitope is a common epitope in smallpox and Zaire 599 strain of monkeypox viruses, but is not present in the Harvey strain smallpox virus. The epitope that reacts with G6C6 MAb is common to the IHD-J strain of vaccinia and the Harvey strain of smallpox viruses, but is not present in monkeypox virus.

Smallpox virus is indispensable for determining the common epitopes shared with other orthopoxviruses; however, it is not available. Using anti-smallpox virus rabbit serum and smallpox patient serum, we tested the apparently type-specific MAbs listed in Table 1 by the CBIA. Six of 16 vaccinia MAbs and 11 of 16 monkeypox MAbs competed with the anti-smallpox serum against vaccinia or monkeypox virus (Table 3). The corresponding epitopes seemed not to be type-specific epitopes but apparently common to smallpox, and the common epitopes between taterapox and monkeypox viruses were included in this group. Taterapox may be a recombinant virus between vaccinia and smallpox.

TABLE 3

EPITOPES IN SMALLPOX VIRUS THAT ARE PARTIALLY COMMON WITH THOSE IN OTHER ORTHOPOXVIRUSES

MAb	CBIA titer*		Smallpox epitope
	Serum of smallpox patient (IHS-73)	Anti-smallpox rabbit serum	
Antigen: IHD-J vaccinia virus			
G 1A10	<40	<40	-
G 8D 9	<40	<40	-
G 3C 1	<40	<40	-
G 3B10	<40	<40	-
G 4G 9	<40	<40	-
G 11D11	90	80	+
G 11C11	420	150	+
G 1B 5	300	210	+
G 6C 6	260	130	+
G 6G11	<40	50	-
G 11B 5	<40	<40	-
G 3F 7	110	70	+
G 8E 2	<40	<40	-
G 9D 9	150	80	+
G 7H 5	<40	<40	-
Antigen: Zaire 599 monkeypox virus			
H 1C10	<40	60	-
H 5A 2	320	<40	+
H 12D 1	520	60	+
I 3A 8	230	<40	+
H 6A11	90	60	+
H 3F 4	230	120	+
H 5B 3	110	70	+
H 6H11	140	70	+
H 12D 5	280	110	+
H 7B 4	<40	80	+
H 5B12	<40	<40	-
B 6F 4	<40	<40	-
C 8B 6	<40	<40	-
D 6A 8	<40	<40	-
H 8G 8	420	160	+
H 12C 1	60	<40	+

* The titers are expressed as the reciprocals of serum dilutions that cause 50% reduction in the binding of added MAb to purified virus.

DISCUSSION

Orthopoxviruses have been classified according to their biological characteristics such as DNA restriction endonuclease maps, their host species, and chemical and physical characteristics. Each species has unique cleavage sites in its DNA, and specific differences in its long terminal repeats. The high sequence homology in two-thirds of the central region of their genomes indicated that many basic characteristics have been conserved among all member viruses, and suggested that the conserved region possesses a high functional value for virus propagation. Since almost all epitopes detected by our MAbs were found in the common surface proteins of the virus, the observed differences in the epitope composition of orthopoxviruses may correlate with minor differences of the DNA make-up in the conserved region.

The type-specific epitopes have been assumed to be unique in each orthopoxvirus, as seen in the strategy to prepare type-specific serum absorption. The absorption method was based on a postulation that each orthopoxvirus adapts well to a specific host species and hence accumulates unique epitopes produced by mutations during the long isolation. For instance, humans are the sole known natural host of smallpox virus. However, the present data show that specificity of orthopoxvirus exists in the uniqueness of its total composition of the partially common epitopes as well as in its unique epitopes. Many more differences were found in the compositions of common

epitopes compared to number of type-specific epitopes between vaccinia and monkeypox viruses. Therefore, most of the species-to-species differences were probably not the result of drifting of antigenicity caused by strain- or species-specific mutations which would produce type- or strain-specific eptiopes, but the result of a mosaic-like rearrangement of common epitopes introduced by recombination. The high cross-reactivity of the polyclonal antibodies against orthopoxviruses is due to this epitope composition.

The stability of epitope sets was indicated by the homology of the epitope composition in strains of vaccinia and monkeypox viruses, and the variability was shown in the mosaic-like differences of common epitope composition among orthopoxvirus species. The type-specific epitopes exist in vaccinia and monkeypox but are scarce compared to the differences in composition of common epitopes. Mere selective preservation of some epitopes from a common progenitor poxvirus did not agree with these findings. In the laboratory, orthopoxviruses can produce recombinants when they are mixedly infected or transfected. Marker rescue takes place easily and many orthopoxviruses can infect various species in addition to the natural host animal. If intraspecies recombination takes place frequently in the wild, it will contribute to the genetic homology or stability among strains. On the other hand, the mosaic-like epitope composition may have been produced by recombination in a variety of common host animals mixedly infected with two orthopoxviruses. The interspecies recombination causes shuffling of genes followed by selective preservation of high functional epitopes, and allows an establishment of species with new epitope compositions. The recombination process may be continuing among wild orthopoxviruses as suggested by the epitope composition of taterapox virus, and by the fact that monkeypox virus had been isolated from at least three species. However, the frequency of such an interspecies event is probably rare enough to maintain the species-specificity of present orthopoxvirus members.

Recombination accompanies the rearrangement of epitope composition. Any orthopoxviruses infectious for human, such as the monkeypox virus in Africa (Jezek *et al.,* 1986), have the potential to become a new threat. Insufficient knowledge of the association of epitope composition and pathogenicity makes it difficult to infer whether highly pathogenic epitope compositions would appear by rearranging the present wild orthopoxvirus epitopes or not. However, the stability of the present epitope combinations suggests that they will require a long time to adapt to a new host, because a total balance of epitope composition, not a small number of type-specific mutations, seems to be important for the process. Eradication of smallpox diminished the source of the epitope set pathogenic for humans, and African monkeypox showed itself to be self-restrictive in humans (Grab *et al.,* 1984). So, the probability of a sudden appearance of a highly pathogenic poxvirus like smallpox by rearrangement is probably low.

ACKNOWLEDGMENTS

We thank Dr. W. K. Joklik and Dr. J. H. Nakano for critical reviewing, and express our appreciation to Dr. Kitamura and Dr. Nakano for supplying various viruses and antisera used in this work. This research was supported by a World Health Organization Technical Assistance Grant, as well as by a Scientific Research Grant of the Ministry of Culture and Science, Japan.

REFERENCES

BAXBY, D. (1975). Identification and interrelationships of the variola/vaccinia subgroup of poxviruses. *Prog. Med. Virol.* **19,** 215–246.

CHO, C. T., and WENNER, H. A. (1973). Monkeypox virus. *Bacteriol. Rev.* **37,** 1–18.

CULLEN, S. E., and SCHWARTZ, B. D. (1976). An improved method for isolation of H-2 and Ia alloantigens with immunoprecipitation induced by protein A-bearing staphylococci. *J. Immunol.* **117,** 136–142.

DUMBELL, K. R., and ARCHARD, L. C. (1980). Comparison of white pock (h) mutants of monkeypox virus with parental monkeypox and with variola-like viruses isolated from animals. *Nature (London)* **286,** 29–32.

ESPOSITO, J. J., and KNIGHT, J. C. (1985). Orthopoxvirus DNA: A comparison of restriction profile and maps. *Virology,* **43,** 230–251.

FENNER, F. (1976). The classification and nomenclature of viruses. Summary of results of meetings of the International committee on taxonomy of viruses in Madrid. September 1975. *J. Gen. Virol.* **31,** 463–470.

FENNER, F. (1985). Poxviruses. *In* "Virology" (Fields, Ed.), pp. 661–703. Raven Press, New York.

GRAB, B., JEZEK, Z., and DIXON, H. (1984). Stochastic model of the interhuman spread of monkeypox. The current status of human monkeypox: Memorandum from a WHO Meeting. *Bull. WHO* **62,** 703–713.

HARPER, L., BEDSON, H. S., and BUCHAN, A. (1979). Identification of orthopoxviruses by polyacrylamide gel electrophoresis of intracellular polypeptides. *Virology* **93,** 435–444.

HUTCHINSON, H. D., ZIEGLER, D. W., WELLS, D. E., and NAKANO, J. H. (1977). Differentiation of variola, monkeypox, and vaccinia antisera by radioimmunoassay. *Bull. WHO* **55,** 613–623.

ICHIHASHI, Y. (1981). Unit complex of vaccinia polypeptide linked with disulfide bridges. *Virology* **113,** 277–284.

ICHIHASHI, Y., and MATSUMOTO, S. (1969). Genetic study of pox group viruses. *Virus* **19,** 155–163.

ICHIHASHI, Y., and OIE, M. (1982). Proteolytic activation of vaccinia virus for the penetration phase of infection. *Virology* **116,** 297–305.

ICHIHASHI, Y., OIE, M., and TSURUHARA, T. (1984). Location of DNA-binding proteins and disulfide-linked proteins in vaccinia virus structural elements. *J. Virol.* **50,** 929–938.

JEZEK, Z., ARITA, I., MUTOMBO, M., DUNN, C., NAKANO, J. H., and SZCZENIOWSKI, M. (1986). Four generations of probable person-to-person transmission of human monkeypox. *Amer. J. Epidemiol.* **123,** 1004–1012.

JOKLIK, W. K. (1962). The purification of four strains of poxviruses. *Virology* **18,** 9–18.

KHODAKEVICH, L., JEZEK, Z., and KINZANZKA, K. (1986). Isolation of monkeypox virus from wild squirrel infected in nature. *Lancet,* 98–99.

KOHLER, G., and MILSTEIN, C. (1976). Derivation of specific antibody-producing tissue and tumor lines by cell fusion. *Eur. J. Immunol.* **6,** 511–519.

LOURIE, B., NAKANO, J. H., KEMP, G. E., and STZER, H. W., (1975). Isolation of poxvirus from an African rodent. *J. Infect. Dis.* **132,** 677–681.

MACKETT, M., and ARCHARD, L. C. (1979). Conservation and variation in orthopoxvirus genome structure. *J. Gen. Virol.* **45,** 683–701.

MULLER, H. K., WITTEK, R., SCHAFFNER, W., SCHUMPERLI, D., MENNA, A., and WYLER, R. (1978). Comparison of five poxvirus genomes by analysis with restriction endonuclease *Hin*dIII, *Bam*HI and *Eco*RI. *J. Gen. Virol.* **38,** 135–147.

NAKANO, J. H. (1985). Poxviruses. *In* "Manual of Clinical Microbiology" (E. H. Lennete, Ed.), pp. 733–741. American Society for Microbiology, Washington, DC.

OIE, M. (1985). Reversible inactivation and reactivation of vaccinia virus by manipulation of viral lipid composition. *Virology* **142,** 299–306.

OIE, M., and ICHIHASHI, Y. (1981). Characterization of vaccinia polypeptides. *Virology* **113,** 263–276.

OIE, M., and ICHIHASHI, Y. (1987). Modification of vaccinia virus penetration proteins analyzed by monoclonal antibodies. *Virology* **157,** 449–459.

ROUMILLAT, L. F., PATTON, J. L., and DAVIS, M. L. (1984). Monoclonal antibodies to a monkeypox virus polypeptide determinant. *J. Virol.* **52,** 290–292.

TOWBIN, H., STAEHELIN, T., and GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.

VOLLER, A., BINDWELL, D., and BARTLETT, A. (1976). Microplate enzyme immunoassay for the immuno-diagnosis of virus infections. *In* "Handbook of Clinical Immunology" (N. R. Rose and H. Friedman, Eds.), pp. 506–512. American Society for Microbiology, Washington, DC.

WITTEK, R. (1982). Organization and expression of the poxvirus genome. *Experientia* **38,** 285–297.