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Differentiation of variola, monkeypox, and vaccinia antisera by radioimmunoassay *

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Poxvirus antisera adsorbed with " homologous " and " heterologous " poxvirus-infected chorioallantoic membranes (CAM) were differentiated by solid-phase radioimmunoassay (RIA). Mixtures of the antiserum dilutions and infected CAM were added directly (without centrifugation) to poxvirus-infected CAM antigens affixed to wells of microtitration plates. The affixed antigens combined with unadsorbed antibodies, and the cross-reactive antigen–antibody complexes were removed by washing. The results showed that adsorption of an antiserum with variola-, vaccinia-, or uninfected-CAM antigen and subsequent reaction of each in RIA with monkeypox- and uninfected-CAM antigens allowed the identification of antivariola, antivaccinia, or antimonkeypox sera.

The variola/vaccinia subgroup of the *Orthopoxvirus* genus consists of a number of biologically and serologically related viruses. Among the members of this subgroup, variola, monkeypox, and vaccinia viruses are important because they cause infections in man (*2*). It is also important in diagnostic virology to be able to differentiate antibodies to each of the viruses.

Members of the subgroup are characterized and categorized primarily by their biological properties. The different viruses show recognizable growth characteristics on chicken embryo chorioallantoic membranes (CAM), rabbit dermis, and in tissue culture.

Recently, about 30 proteins each for variola, vaccinia, and monkeypox viruses (*5*) were enumerated by polyacrylamide gel electrophoresis. Most of these proteins are shared by the members of the subgroup; however, high resolution electrophoresis showed characteristic, unique proteins for each of these subgroup members.

Because of multiple specificities of antibodies directed towards component antigens, identifying antisera raised against the individual viruses has been difficult. Results of neutralization tests, haemagglutination inhibition (HI) tests and immunodiffusion tests (*4, 7, 9, 15, 22*) show that antisera to the various subgroup members are strongly cross-reactive with heterologous viruses within the subgroup. In spite of the cross-reactivity, adsorption studies have allowed the unique antigens among the members of the poxvirus subgroup to be defined (*1, 4, 9*).

Gispen & Brand-Saathof (*9*) and Esposito et al. (*4*) used selective adsorption to prepare specific antisera that permit identification of variola, vaccinia, or monkeypox viruses. Multiple adsorptions of potent antisera with heterologous viruses yielded sera that produced one or two immunodiffusion precipitin lines with homologous viruses. The precipitin lines obtained with adsorbed sera probably involve strain-specific antigens not shared by other related viruses of the subgroup. The antisera have been used to identify antigenically related viruses. Recently, Gispen et al. (*10*) described an immunofluorescent assay in which they differentiated vaccinia and monkeypox antisera after adsorption with homologous and heterologous antigens. They did not, however, include antiserum to variola virus.

An alternative procedure involved adsorbing patients' sera with poxvirus antigens and subsequently characterizing residual antibodies with a sensitive

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radioimmunoassay (RIA), which apparently allowed poxvirus antisera identification. Ziegler et al. (*27*) described an RIA for detecting antibodies to poxviruses that was more sensitive than complement fixation (CF), HI, or plaque reduction neutralization (PRN). In this study we adsorbed antisera with homologous and heterologous antigens, and determined the specific reactivity of the residual antibody activity by RIA. It was possible to differentiate antisera to closely related poxvirus strains by their adsorption patterns.

MATERIALS AND METHODS

Virus strains

Poxvirus strains were obtained from the Viral Exanthems Branch, CDC, as CAM passage materials. Except for vaccinia, the viruses were originally isolated from crust materials from infected patients or infected nonhuman primates. The strains used are listed below.

— Wyeth strain of vaccinia (Vac) was originally obtained from Wyeth Laboratories, Philadelphia, PA.

— Harvey (Har), a variola (Var) major strain, obtained from Professor K. R. Dumbell, was isolated from a smallpox outbreak in Middlesex, England, 1944.

— African smallpox (ASP), a variola major strain, was isolated from a patient in Sierra Leone in 1968 (CDC No. V68-I-258) (*13*).

— Chimp 9 (Ch-9) virus, a whitepoxvirus strain (variola-like) was isolated from an apparently healthy chimpanzee in Zaire in 1971 (CDC No. V71-I-224) (*16*).

— Utrecht monkeypox virus (MMP) was isolated from an orangutan in Rotterdam, Netherlands in 1965 during a poxlike epizootic outbreak (*20*).

— Human monkeypox virus (HMP), a monkeypox virus strain, was isolated from a patient in Sierra Leone in 1970 (CDC No. V70-I-266) (*13*).

To confirm the identity of the poxviruses used in this study, the Viral Exanthems Branch, CDC, tested coded samples of the poxvirus-infected CAM preparations. Identifications were made on the basis of the pock characteristics on CAM as described by Nakano (*17*).

Antigen preparation

Antigens for RIA and selective adsorption of sera were prepared by inoculating 12-day-old chicken CAM with 0.1-ml aliquots of dilutions (usually 10^{-2}) of the virus preparations as described by Nakano & Bingham (*18*). Uninfected control CAM was prepared by inoculating the eggs with McIlvaine's buffer (*18*). After 3 days of incubating the eggs at 35.5°C the membranes were harvested and frozen once at —70°C. Membrane antigens were prepared by thawing the membranes and homogenizing in a Sorvall omnimixer (three 1-min cycles) at 4°C. The suspensions were centrifuged for 10 min at 900 *g*. The supernatant was collected and stored in small aliquots at —70°C. Each viral antigen preparation was titrated on CAM and the titre was reported as pock-forming units per millilitre (PFU/ml). Generally, antigen preparations containing more than 10^7 PFU/ml were satisfactory for RIA antigen or adsorption antigen. The protein concentrations of the RIA antigens were determined (*14*) and were adjusted to 10 mg/ml before use.

Antiserum preparations

Hyperimmune antisera were prepared in albino rabbits by the Viral Exanthems Branch, CDC, as described by Esposito et al. (*4*). Each poxvirus antigen was prepared in primary rabbit kidney tissue culture cells. When the cytopathic effect was 100%, infected cells in each 950-ml (32-oz) prescription bottle were scraped from the glass, centrifuged, and resuspended in 3 ml of McIlvaine's buffer. The virus was dissociated from the cells by freezing and thawing. All viral antigens had titres greater than 10^7 PFU/ml on CAM. Each rabbit was initially inoculated with a homogenate containing equal volumes of poxvirus antigen and Freund's complete adjuvant. 0.25 ml of this viral emulsion was inoculated into both front footpads and 0.5 ml into the right hindleg muscle. A booster inoculum containing only the poxvirus antigen was given 24 days later. 1 ml was given subcutaneously in the neck and 0.5 ml in the right leg muscle. The rabbits were exsanguinated 18 days after the booster inoculations.

Antisera were prepared to Wyeth Vac virus (antiVac; CF titre $\geqslant$ 512, HI titre = 320, PRN titre = 25 000), ASP virus (antiVar; CF titre = 512, HI titre = 160, PRN titre = 1800), Ch-9 virus (antiCh-9; CF titre = 1024, HI titre = 640, PRN titre = 3200), MMP virus (antiMMP; CF titre = 512, HI titre = 640, PRN titre = 9000), and HMP

virus (antiHMP; CF titre = 512, HI titre = 320, PRN titre = 8000). Aliquots of the antisera were prepared and frozen at −70°C.

Standardization of poxvirus antigens for indirect RIA

Because optimum amounts of the reagents are necessary to achieve high sensitivity and reproducibility in RIA, the optimum concentration of each primary antigen and ^{125}I-antirabbit globulin used in the RIA was determined as described by Lee et al. (*12*). Optimum amounts of each antigen, including uninfected CAM, were determined by reacting an antiserum diluted 1 : 10 with various concentrations of antigen. Controls containing diluent instead of antiserum were included for each antigen concentration.

After a secondary reaction with the optimum concentration (*11*) of ^{125}I-antirabbit globulin, the radioactivities (cpm) of the different components were determined. Binding ratios were determined for each antigen concentration by the following formula:

$$(X - Y + Z)/Z$$

where X = (cpm) antiserum against homologous antigen
Y = (cpm) antiserum against uninfected antigen
Z = (cpm) diluent control.

The data were evaluated by plotting the binding ratios on the ordinate and the logarithm (log) of the protein concentration on the abscissa (Fig. 1). For each poxvirus antigen shown (Vac, Har, Ch-9, and UMP), the highest ratios were obtained with 15.6 μg of protein per well. Hence antigen preparations with this concentration of protein were used in the RIA procedures. Although the figure represents single lots of antigen preparations, results of evaluating subsequent lots in an identical manner were comparable.

Adsorption procedure

Twofold (or threefold) antiserum dilutions prepared in phosphate buffered saline (PBS) containing 20% fetal calf serum and 0.04% sodium azide (PBS diluent) were transferred to separate tubes in 0.2-ml aliquots, and an equal volume of infected-CAM or uninfected-CAM adsorption antigen in PBS was added to each dilution. In preliminary experiments, PBS diluent was also added to a series of serum dilutions as controls. The serum titrations were mixed and held at 4°C for 16 to 18 h. Immediately before they were added to the antigen-containing microtitration plates, the serum dilutions were incubated for 30 min at 36°C.

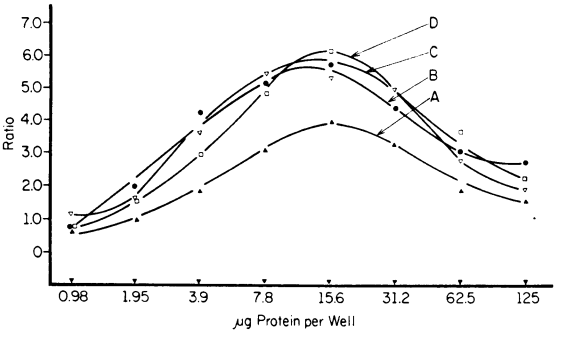

Fig. 1. Poxvirus antigen titrations. Various concentrations of each antigen were reacted with a single (1 :10) dilution of "homologous" antiserum. Ratios were calculated as indicated above. (A) Har antigen against antiVar serum; (B) Vac antigen against antiVac serum; (C) Ch-9 antigen against antiCh-9 serum; (D) MMP antigen against antiMMP serum.

RIA procedure

The indirect RIA procedure for poxviruses was previously described by Ziegler et al. (*27*). An optimal concentration of infected and uninfected CAM antigen materials was prepared in PBS and added to the wells of microtitration plates. The plates were air-dried overnight at room temperature, and fixed for 15 min with 10% buffered formaldehyde. Sequential serum dilutions (0.025 ml aliquots) adsorbed with homologous or heterologous antigens were added to both homologous and heterologous CAM antigens and uninfected CAM antigen and allowed to incubate for 2 h at 36°C. After the plates were washed with PBS, an optimal concentration of ^{125}I-antirabbit globulin (*11*) was added to each well. The plates were incubated for 1 h at 36°C and washed with PBS.

Each sample was cut from the microtitration plate and was counted in a gamma scintillation spectrometer. End-point dilution factors (titres) were determined as described by Hutchinson & Ziegler (*11*). Titres were obtained by plotting the log of the specifically bound radioactivity [(cpm) antiserum against infected CAM—(cpm) antiserum against uninfected CAM] + (cpm) diluent control on the ordinate and the log of the reciprocal antiserum dilutions on the abscissa. The antiserum titration curves were interpolated or extrapolated to the reciprocal antiserum dilution at which the bound radioactivity equalled twice the diluent control.

RESULTS

Antiserum titrations with homologous antigens

Viruses of the family Poxviridae contain multiple common protein components. Thus, antibodies prepared against one of the viruses of the group have multiple specificities which cross-react with related viruses. Before examining the antigenic relationships among closely related poxviruses, we titrated each antiserum against its homologous antigen, comparing the results with those obtained when the antiserum was titrated against heterologous antigens. Titration curves (log–log plots) of Vac–antiVac, Ch-9–antiCh-9, MMP–antiMMP, and Har–antiVar are shown in Fig. 2. Except for the Har–antiVar curve, each titration curve was linear and essentially parallel to the others. The plot of Har–anti Var was

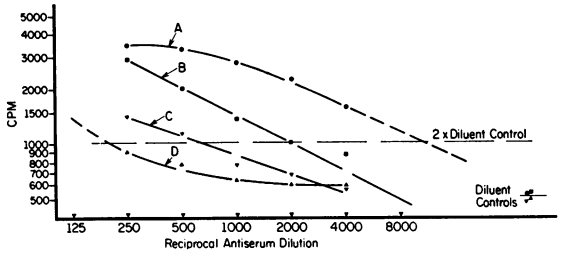

Fig. 2. " Homologous " antiserum titrations. The horizontal lines represent control (cpm) ×2. (A) Har antigen against antiVar serum; (B) Vac antigen against antiVac serum; (C) Ch-9 antigen against antiCh-9 serum; (D) MMP antigen against antiMMP serum.

slightly curvilinear; however, with repeated titrations the end-point titre of the antiserum was reproducible. Antiserum titrations against both homologous and heterologous antigens consistently yielded curves of similar slopes.

Selection of antigen concentrations for adsorption

Before attempting to adsorb antibodies from antisera with related antigens, we determined the quantity of homologous antigen required to remove virtually all antibodies from an antiserum. Each antiserum was adsorbed with 0.1, 1.0, or 10 mg of antigen per millilitre before it was titrated with homologous antigen. The titration curves obtained for antiCh-9 serum (Fig. 3) show that larger amounts of antibody against Ch-9 virus were removed as the quantity of homologous adsorbent antigen was increased. The highest concentration (10 mg/ml) of adsorbent antigen removed virtually all of the antibodies. The end-point titres were <2.3%, 5.9%, and 16.2% of the unadsorbed titre values after adsorption with 10, 1.0, and 0.1 mg of the antigen per millilitre, respectively.

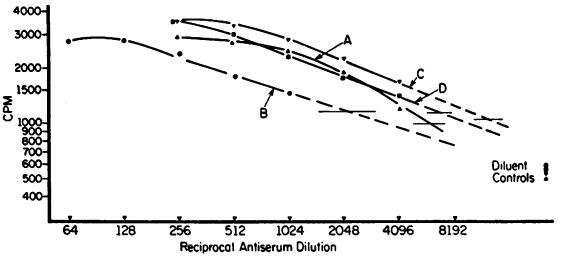

Fig. 3. Adsorption of antiCh-9 serum with various concentrations of Ch-9 antigen. (A) No adsorption, or adsorption with each of the following: (B) 0.1 mg/ml; (C) 10 mg/ml; (D) 10 mg/ml of Ch-9 antigen.

Like the antiCh-9 serum adsorptions, antiMMP, antiVac, and antiVar serum adsorptions with their homologous antigens reduced the antiviral antibodies in amounts proportional to the amount of adsorbent used. Adsorption with 10 mg of homologous antigens per millilitre removed more than 95% of the antibody activity of each antiserum (Table 1). In subsequent experiments with antisera adsorbed with their homologous antigens (10 mg/ml) the antibody titres were consistently reduced by more than 95%. Although we were unable to remove antibody activity completely by a single adsorption with 10 mg of the adsorbent antigens per millilitre, it was impractical to use higher concentrations.

Adsorption of antisera with uninfected CAM

Poxvirus antigens used in these investigations were crude suspensions prepared by homogenizing poxvirus-infected CAM. Thus, in addition to viral antigens, the preparations contained large amounts of host tissue that could react nonspecifically. The effect of adsorption of antisera with uninfected CAM homogenates was therefore examined. As shown in Table 2, the homologous titres of CAM-adsorbed antisera were 1.3–3.9 times higher than the titres obtained with the antisera without adsorption with CAM.

The increased titres, after adsorption, were partially attributed to decreased binding of ^{125}I-antirabbit globulin to the uninfected-CAM controls (nonspecific). However the major reason for increased

Table 1. Adsorption of poxvirus antisera with various concentrations of homologous antigens

Antigen	against antiserum	Adsorbing antigen concentration						
		Unadsorbed	10 mg/ml		1.0 mg/ml		0.1 mg/ml	
		Titre [a]	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]
UMP	antiUMP	8 000	<250	<3.1	580	7.2	2 000	25
Vac	antiVac	1 680	<64	<3.8	88	5.2	560	33
Har	antiVar	5 500	<250	<4.5	285	5.2	1 300	24
Ch-9	antiCh-9	10 800	<250	<2.3	640	5.9	1 750	16

[a] Titre is the end-point dilution factor.
[b] Percentage of original (unadsorbed) antibody titre.

titres was the enhanced binding of ^{125}I-antirabbit globulin to the poxvirus-infected antigens (specific). Because this phenomenon was reproducible, adsorption of antisera with uninfected CAM antigen was adopted as a part of the standard adsorption procedure.

Homologous and heterologous antiserum adsorptions

The antigenic relationships among five orthopoxviruses, Ch-9, Har, ASP, MMP, and Vac, were evaluated by RIA. Five antisera, antiVar, antiCh-9, antiVac, antiMMP, and antiHMP, were each adsorbed with six antigens, uninfected CAM, Ch-9, Har, ASP, MMP, and Vac viruses, and were tested by RIA for residual antibodies reactive with both homologous and heterologous antigens.

The results of the adsorptions of antiVar, antiVac, and antiMMP sera are shown in Tables 3-5. Generally, the CAM-adsorbed antisera had the highest titres with homologous antigens. In some cases, however, using this measurement as the sole criterion for differentiating the antisera could lead to equivocal or erroneous assignment of strain specificity. As an example, antiVar serum reacted with MMP antigen yielded a higher titre than it did when it was reacted with ASP antigen. This fact indicates the importance of considering the titres obtained after adsorption.

Table 2. Antisera adsorbed with normal CAM antigen

Antigen	Antiserum	Unadsorbed	CAM adsorbed [a]	
		Titre [b]	Titre [b]	Fold increase [c]
Ch-9	antiCh-9	10 076	12 660	1.3
Har	antiVar	NT [d]	6 498	–
Vac	antiVac	853	3 292	3.9
MMP	antiMMP	4 485	6 884	1.5
MMP	antiHMP	2 202	4 123	1.9

[a] 10 mg of uninfected CAM antigen per millilitre used for adsorption.
[b] Titre is the end-point dilution factor.
[c] Fold increase in titre as compared to the unadsorbed titre.
[d] NT=not tested.

The results of homologous and heterologous adsorption of antiVar serum are shown in Table 3. Adsorption of this antiserum with Ch-9, Har, or ASP antigen removed essentially all antibody activity to Ch-9, Har, ASP, and Vac antigen. However low residual antibody activity which was reactive with MMP antigen remained after this adsorption. Conversely, adsorption of antiVar serum with MMP antigen yielded a serum with substantial antibody activity (8.0%–18.8%) to Ch-9, Har, and ASP antigens. The adsorbed antiserum had low antibody activity to MMP antigen (0.4%) and Vac antigen (2.8%). The same antiserum (antiVar) adsorbed with Vac antigen resulted in a serum with antibody activity to Ch-9, Har, ASP, and MMP viruses, but none to Vac virus.

Next, similar adsorptions of antiVac serum were examined (Table 4). After the serum had been adsorbed with Ch-9, Har, or ASP, most of the antibody activity to these antigens was removed whereas considerable antibody activity to MMP and Vac viruses remained (5.5%–11.4%). Adsorption of antiVac serum with MMP antigen yielded an antiserum with antibody activity to Ch-9, Har, ASP, and Vac viruses (5.8%, 5.0%, 4.8%, and 3.7%, respectively) but completely removed the antibody activity to MMP antigen. After antiVac serum was adsorbed with homologous antigen, only very low residual

Table 3. Homologous and heterologous antiserum titrations of antiVar serum

Antiserum adsorbed with	Antigen reacted with									
	Ch-9		Har		ASP		MMP		Vac	
	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]
CAM	5510		6493		5062		5598		2914	
Ch-9	9	0.2	38	0.6	34	0.7	108	1.9	<8	<0.3
Har	27	0.5	51	0.8	41	0.8	95	1.7	<8	<0.3
ASP	17	0.3	25	0.4	9	0.2	52	0.9	<8	<0.3
MMP	533	9.7	1222	18.8	403	8.0	25	0.4	83	2.8
Vac	392	7.1	494	7.6	178	3.5	257	4.6	<8	<0.3

[a] Titre is the end-point dilution factor. Underlined values indicate adsorptions or reactions with homologous antigens.
[b] Percentage of CAM-adsorbed antibody activity remaining.

Table 4. Homologous and heterologous antiserum titrations of antiVac serum

Antiserum adsorbed with	Antigen reacted with									
	Ch-9		Har		ASP		MMP		Vac	
	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]
CAM	2081		2351		1444		2491		3400	
Ch-9	<8	<0.4	<8	<0.3	<8	<0.6	225	9.0	386	11.4
Har	16	0.8	<8	<0.3	<8	<0.6	138	5.5	269	7.9
ASP	40	1.9	23	1.0	<8	<0.6	182	7.3	223	6.6
MMP	120	5.8	118	5.0	70	4.8	<8	<0.3	127	3.7
Vac	39	1.9	22	0.9	37	2.6	11	0.4	62	1.8

[a] Titre is the end-point dilution factor. Underlined values indicate adsorptions or reactions with homologous antigens.
[b] Percentage of CAM-adsorbed antibody activity remaining.

Table 5. Homologous and heterologous antiserum titrations of antiMMP serum

Antiserum adsorbed with	Antigen reacted with									
	Ch-9		Har		ASP		MMP		Vac	
	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]
CAM	1163		1111		884		3702		1807	
Ch-9	<8	<0.7	<8	<0.7	<8	<0.9	1277	34.5	35	1.9
Har	<8	<0.7	<8	<0.7	<8	<0.9	840	22.7	32	1.8
ASP	<8	<0.7	<8	<0.7	<8	<0.9	550	14.9	26	<0.4
MMP	<8	<0.7	<8	<0.7	<8	<0.9	49	1.3	<8	<0.4
Vac	<8	<0.7	<8	<0.7	<8	<0.9	740	20.0	<8	<0.4

[a] Titre is the end-point dilution factor. Underlined values indicate adsorptions or reactions with homologous antigens.
[b] Percentage of CAM-adsorbed antibody activity remaining.

Table 6. Homologous and heterologous antiserum titrations of antiCh-9 serum

Antiserum adsorbed with	Antigen reacted with									
	Ch-9		Har		ASP		MMP		Vac	
	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]
CAM	7911		7809		NT [c]		5809		4916	
Ch-9	<8	<0.1	24	0.3	NT		<8	<0.1	<8	<0.2
Har	15	0.2	32	0.4	NT		11	0.2	11	0.2
ASP	<8	<0.1	35	0.4	NT		<8	<0.1	<8	<0.2
MMP	1172	14.8	1050	13.4	NT		<8	<0.1	95	1.9
Vac	401	5.1	398	5.1	NT		43	0.7	10	0.2

[a] Titre is the end-point dilution factor. Underlined values indicate adsorptions or reactions with homologous antigens.
[b] Percentage of CAM-adsorbed antibody activity remaining.
[c] NT=not tested.

antibody activity remained to each of the antigens. The residual activity ranged from 0.9% to 2.6% of the CAM-adsorbed titres with no indication of specificity for any of the antigens. Residual activity was highest with Vac antigen, probably reflecting incomplete adsorption of the antibodies.

Finally, homologous and heterologous adsorptions of antiMMP serum were studied (Table 5). When antiMMP serum was adsorbed with either Ch-9, Har, or ASP antigens, all antibody activity to these three poxviruses was removed. Antibody activity to MMP virus remained high in the adsorbed serum (14.9% to 34.5%), but very low residual activity to Vac antigen remained (<0.4–1.9%). Adsorption of antiMMP serum with MMP antigen completely removed antibody activity to each of the antigens except MMP. The low residual antibody activity (1.3%) to MMP antigen probably reflects incomplete adsorption and is of no consequence. Adsorption of antiMMP serum with Vac antigen produced an antiserum which was monoreactive with MMP antigen. The monoreactive antibody activity remaining after adsorption was 20.0% of the original antibody activity.

Comparison of antiVar and antiCh-9 sera

Ch-9-virus and variola-virus strains cannot be differentiated by conventional serological procedures and growth characteristics. Further, the RIA reactivity patterns of antisera prepared against the two viruses and adsorbed with homologous and heterologous viruses are basically the same (Tables 3 and 6). However, when the antiVar serum was adsorbed with Ch-9, Har, or ASP antigen, it had low residual antibody titres of 108, 95, and 52, respectively, when titrated against MMP antigen. Unlike the adsorptions of antiVar, adsorption of antiCh-9 serum with Ch-9, Har, or ASP removed all antibodies which reacted with MMP virus in the RIA. This minor discrepancy between the reactions of the two antisera appears to reflect quantitative rather than qualitative differences. Such differences could be attributed to incomplete adsorption caused by differences in the avidity of the two antisera.

Comparison of antiMMP and antiHMP sera

Although the Utrecht strain of MMP virus was isolated from an orangutan in Rotterdam and HMP was isolated from a human patient in Sierra Leone, the two viruses are thought to be identical (*13*, *20*). Several laboratories have used conventional techniques to investigate and confirm their identities. The two isolates have identical growth characteristics (*13*, *15*) and cannot be differentiated by conventional serological procedures (*22*). Likewise, when adsorbed with homologous and heterologous viruses and tested by RIA, antiMMP and antiHMP sera had similar adsorption patterns (Tables 5 and 7). In fact, despite minor quantitative differences in the adsorption studies, the two were qualitatively identical.

Identification of a reaction scheme for simple separation of poxviruses into groups

The usefulness of a serological procedure depends upon its simplicity and sensitivity. The described RIA adsorption procedure is both easy and sensitive.

Table 7. Homologous and heterologous antiserum titrations of antiHMP serum

Antiserum adsorbed with	Antigen reacted with									
	Ch-9		Har		ASP		MMP		Vac	
	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]	Titre [a]	% [b]
CAM	NT [c]		811		734		1816		862	
Ch-9	NT		13	1.6	9	1.2	285	15.7	60	7.0
Har	NT		9	1.1	10	1.4	293	16.1	36	4.2
ASP	NT		<8	<1.0	<8	<1.1	126	6.9	20	2.3
MMP	NT		<8	<1.0	17	2.3	28	1.5	23	2.7
Vac	NT		<8	<1.0	<8	<1.1	156	8.6	<8	<0.9

[a] Titre is the end-point dilution factor. Underlined values indicate adsorptions or reactions with homologous antigens.
[b] Percentage of CAM-adsorbed antibody activity remaining.
[c] NT=not tested.

Adsorptions of sera with antigens of each poxvirus group and subsequent testing of the adsorbed sera by RIA with each antigen allow identification of serum specimens. However the multiple steps make the utility of the method questionable. To determine whether a simple adsorption scheme could be established for identifying routine diagnostic specimens, we examined the results in Tables 3–7 for definitive steps for differentiating antisera.

Identifying the specificity of a poxvirus antiserum depends upon evaluating the residual antibody activity (percentage of original) remaining after adsorption. Evaluation of the adsorption data revealed that the group identity of antisera could be determined by adsorbing individual aliquots of an unknown serum with uninfected CAM, Ch-9 (or Var), and Vac antigens and then allowing it to react with MMP and uninfected CAM antigens in RIA.

When antiVar serum is adsorbed with Var antigen, a serum with very low or negligible antibody activity to MMP antigen is produced. When a second sample of the same serum is adsorbed with Vac antigen, residual antibody activity to MMP is likewise low. An antiVac serum had moderate residual antibody activity to MMP virus when adsorbed with Var antigen and low activity when adsorbed with Vac antigen. Conversely, an antiMMP or antiHMP serum had moderately high residual activity after adsorption with either Var or Vac antigens. Thus antisera prepared against either variola, vaccinia, or monkeypox viruses can be identified by a simple adsorption procedure.

DISCUSSION

The incidence of smallpox infection in man has rapidly diminished since the inception of the smallpox eradication programme in 1966. There have, however, been sporadic smallpox cases in Somalia and Kenya during 1977 (*24*). Additionally, three documented human monkeypox infections have been reported as recently as February and March 1977 (J. H. Nakano, WHO Collaborating Centre for Smallpox and Other Poxvirus Infections, unpublished data).

Human monkeypox virus is biologically indistinguishable from viruses isolated from clinically ill monkeys (*6*, *13*, *16*) or from apparently healthy monkeys (*15*, *16*). In addition, a virus that closely resembles variola (*8*, *16*) has been isolated from a chimpanzee and a cynomolgus monkey. The presence of these viruses in the animal population and the occurrence of sporadic human infections requires that smallpox surveillance be continued. Future surveillance of human and animal populations for smallpox and monkeypox infections in remote areas of the world will be made after obvious lesions have disappeared. Hence, virus isolation will be impossible, and only serum specimens will be collected. Therefore, a method for identifying poxvirus strain-specific antibodies will be necessary for successful retrospective studies.

Serological surveys can be used only as a measure of the immunological status of a population to the *Orthopoxvirus* genus. Until recently, differentiation

among closely related poxvirus antibodies has depended upon differences in the antibody titres to homologous and heterologous viruses (*10*, *22*).

Serological studies have shown repeatedly that the orthopoxviruses are antigenically related (*1*, *3*, *7*, *19*, *23*, *25*, *26*). Nevertheless, immunodiffusion procedures have revealed soluble antigens not shared by all of the orthopoxviruses (*7*, *19*, *21*). Specific antisera have also been prepared by adsorption with heterologous antigens (*4*, *9*). These antisera were capable of differentiating vaccinia-, variola-, and monkeypox-related viruses.

Members of the *Orthopoxvirus* genus possess antigens not found in other strains of the group (*5*). As a result, antisera raised against a specific strain would also have unique antibodies, as the results of Gispen & Brand-Saathof (*9*) and Esposito et al. (*4*) have shown. Their results suggest that selective adsorption of common antibodies from sera and subsequent identification of the residual antibodies could provide a means of identifying antisera of unknown specificity. In fact, Gispen et al. (*10*) recently showed that adsorbed antisera to monkeypox virus and vaccinia virus can be differentiated; antisera to variola virus were not included in the study, however.

The relationships among antisera prepared against poxviruses revealed by RIA indicate that adsorption of antisera with heterologous antigens did not uniformly produce monospecific reactivity; however adsorption with an antigen removed antibodies reactive with that antigen (i.e., homologous). The cross-reactions with heterologous viruses do not preclude the presence of monoreactive antigens because complete adsorption of cross-reactive antigens was not attempted.

This study confirmed that (*a*) there is an antigen group on variola virus that is common to vaccinia virus but not to monkeypox virus (*9*), and (*b*) monkeypox virus contains a unique antigen group (*9*).

Residual antibody activities were observed which seemed to reflect two antigen groups that have not been reported previously (*4*, *9*). Variola and monkeypox viruses have an antigenic group in common that is not found in vaccinia; and vaccinia and monkeypox viruses have antigen(s) in common that variola virus does not have.

The adsorption patterns of antibody produced to chimp-9 virus and African smallpox virus were nearly identical, as were those produced to monkey monkeypox and human monkeypox viruses. Thus these adsorption studies reconfirm that the following pairs of virus isolates are closely similar if not identical: chimp-9 virus and variola viruses; and isolates of monkeypox viruses obtained from monkeys and those obtained from humans.

The results of this study using hyperimmune rabbit sera show that the antigenic relationship among monkeypox, variola, and vaccinia allows a relatively simple adsorption scheme to be established. To determine the specificity of the antibodies, adsorbed antisera must be reacted only with crude monkeypox virus-infected and uninfected chorioallantoic membranes. The use of this adsorption scheme for differentiating the poxvirus specificity of diagnostic sera is currently being investigated.

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RÉSUMÉ

DIFFÉRENCIATION PAR LA MÉTHODE RADIO-IMMUNOLOGIQUE DES IMMUNSÉRUMS ANTI-VARIOLE, ANTI-MONKEYPOX ET ANTI-VACCINE

L'identification des anticorps à l'égard des divers virus du sous-groupe variole/vaccine du genre *Orthopoxvirus* est malaisée en raison d'importantes réactions croisées. Cette identification est d'un intérêt considérable du point de vue du diagnostic virologique, mais la mise au point de méthodes permettant une différenciation indubitable des anticorps à l'égard de souches spécifiques de poxvirus s'est révélée difficile. Dans le présent travail, on a évalué une méthode de différenciation des immunsérums à l'égard des virus de la variole, de la vaccine, ainsi que des virus du monkeypox de l'homme et du singe. L'adsorption des immunsérums à l'égard de chacune de ces souches virales par des antigènes de poxvirus spécifiques et la caractérisation ultérieure des anti-

corps résiduels par dosage radio-immunologique (RIA) ont fourni un moyen d'identifier les immunsérums contre les poxvirus. Des dilutions de ces sérums anti-poxvirus ont été adsorbées avec des préparations d'antigènes provenant de membranes chorio-allantoïdiennes (MCA) infectées de poxvirus « homologues » ou « hétérologues »; ces dilutions ont ensuite été ajoutées directement (sans centrifugation) à des antigènes de MCA infectées de poxvirus, fixés à des cupules de plaques pour microtitrage. Les anticorps non adsorbés restants étaient libres de réagir avec l'antigène fixé à la phase solide, alors que les anticorps à réactivité croisée, adsorbés par la MCA infectée de virus, n'étaient pas libres de le faire.

En raison de la communauté antigénique étendue chez les virus de ce groupe, les adsorptions ne produisaient pas invariablement des immunsérums ayant une réactivité « monospécifique » à l'égard de l'antigène homologue. Les adsorptions ont confirmé la présence de deux groupes antigéniques antérieurement décrits: 1) le virus de la variole et le virus de la vaccine qui possèdent un groupe antigénique commun; et 2) le virus monkeypox qui a un groupe antigénique propre. D'après ces recherches, il semble en outre qu'il existe deux autres relations antigéniques, ainsi: 1) les virus de la variole et du monkeypox ont un groupe antigénique commun que ne possède pas le virus de la vaccine; et 2) les virus de la vaccine et du monkeypox ont un groupe antigénique commun que ne possède pas le virus variolique.

Les résultats de l'adsorption des anticorps à l'égard du virus chimp-9 et du virus de la variole (variole africaine) étaient presque identiques; il en était de même dans le cas des anticorps produits à l'égard des virus de monkeypox simien et du monkeypox humain. Ainsi, ces études confirment une fois de plus la grande similitude, sinon l'identité, du virus chimp-9 et du virus de la variole, d'une part, ainsi que des virus monkeypox isolés des singes et de l'homme, d'autre part.

La méthode d'adsorption a fourni un moyen pratique de différencier les immunsérums à l'égard des virus de la variole, du monkeypox et de la vaccine. Il n'est pas nécessaire d'adsorber les échantillons de sérums avec chacun des adsorbants viraux et de déterminer les titres résiduels contre chacun des virus du sous-groupe. Les immunsérums ont été d'abord adsorbés par de la MCA non infectée, du virus de la vaccine ou de la variole; puis on les a fait réagir avec de la MCA non infectée et de la MCA infectée de virus monkeypox en RIA. Les immunsérums à l'égard de chacun de ces virus ont présenté une activité anticorps résiduelle caractéristique à l'égard du virus monkeypox après adsorption.

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