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Preparation and Evaluation of a Noninfectious Monkey Pox Virus Vaccine

RICHARD G. OLSEN,* JAMES R. BLAKESLEE, LARRY MATHES, AND JAMES H. NAKANO

Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio, and the Viral Exanthems Branch, Virology Division, Bureau of Laboratories, Center for Disease Control, Atlanta, Georgia 30333*

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Monkey pox virus was mechanically disrupted by low temperature and high pressure into soluble and insoluble fractions. Soluble fractions elicited virus-neutralizing antibodies (1:20 to 1:160) in rabbits, whereas the insoluble (in saline) fractions did not (<1:5). No infectious virus was detected after the disruption procedure. Rhesus monkeys immunized with the soluble fraction elicited virus-neutralizing (1:1,200), complement-fixing (1:16), and hemagglutinating-inhibiting (1:80 to 1:160) antibody titers and were completely protected against monkey pox virus-induced disease. This model of monkey pox virus subunit vaccine preparation may prove to be useful in developing an efficacious noninfectious vaccinia vaccine for use in high-risk individuals.

Although vaccination against smallpox with live vaccinia virus is a relatively safe procedure, serious complications have been reported to occur, i.e., generalized vaccinia in immunosuppressed patients, postvaccinal encephalitis, eczema vaccinatum in young children, and a rare but usually fatal dermal complication, vaccinia gangrenosa (12). The preparation of a noninfectious subunit smallpox vaccine containing immunologically effective viral components would be a useful product for these high-risk individuals.

Previous studies with poxvirus subunit vaccines prepared by disrupting vaccinia virus with potassium hydroxide or sodium dodecyl sulfate effectively blocked neutralization of intact vaccinia virus and elicited virus-neutralizing (VN) antibodies in the sera of immunized rabbits (14). Whereas there was no correlation between the titer of neutralizing antibodies and their resistance to challenge by live virus, animals vaccinated with live virus were more resistant to infection than those immunized with subunit vaccine, although their sera had lower titers of VN antibodies (1). Further studies, in which the structural components were analyzed by polyacrylamide gel electrophoresis, suggest that more than a single kind of major protein is localized in the outer virion coat, but these were not further characterized as responsible for eliciting VN antibody (5). In previous studies, Yaba (10) poxviruses mechanically disrupted by high pressure-low temperature treatment (X-press) released structural proteins that elicited VN antibodies. This led us to investigate a method of preparing a subunit vaccine from monkey pox virus (MPV).

In the study reported here MPV particles were mechanically disrupted and separated into soluble and insoluble fractions. Each MPV fraction was evaluated for its ability to (i) elicit antibody production to MPV in rabbits and monkeys, and (ii) protect immunized rhesus monkeys against monkey pox disease.

MATERIALS AND METHODS

Virus propagation and vaccine preparation. The von Magnus (Copenhagen) strain of MPV was propagated in LLC-MK_2 cells as described by Hall et al. (2).

Two different virus preparations were used to produce mechanically disrupted MPV vaccine. These included: a high-speed pellet of MPV and density gradient-purified virus. The former was prepared by centrifuging stock virus preparations at 60,000 × *g* for 30 min. For purification, MPV pellets were suspended in 0.001 M tris(hydroxymethyl)-aminomethane-ethylenediaminetetraacetate buffer and purified on a 10 to 40% sucrose gradient by methods described by Joklik (6).

MPV (virus pellets or purified virus) was mechanically disrupted by an X-press (Ab Biox-Nacka, Sweden) by methods of Olsen and Yohn (10). After mechanical disruption, the preparation was centrifuged at 60,000 × *g* for 30 min, and the soluble and insoluble fractions were separated. The insoluble (in saline) residue remaining after disruption was hydrolyzed with 2.5% sodium dodecyl sulfate-0.7 M urea-1.5% 2-mercaptoethanol. The saline-soluble and -insoluble fractions were referred to as XP and SUM, respectively. When used for immunization of

rabbits or rhesus monkeys, the protein concentration of each fraction was adjusted to 10 mg/ml as determined by the Lowry method (7).

Virus infectivity assay. MPV infectivity was quantitated by a modification of a procedure described by Munyon et al. (9). Briefly, sonically treated preparations of MPV were diluted in serum-free minimal essential medium (MEM), and 0.2 ml of these dilutions was inoculated on LLC-MK_2 cells growing in 60-mm petri dishes and allowed to adsorb for 1 h at 36°C. A 5.0-ml amount of MEM plus 2% fetal bovine serum (FBS) and 80 μg of trypsin (1:250; Difco, Detroit, Mich.) per ml were added to each dish and incubated for 48 h. The medium was aspirated, and the cells were fixed with 2 ml of absolute methanol and stained with crystal violet. The number of plaques was found to be proportional to virus concentration.

Immunization procedures. (i) Rabbits. Five New Zealand rabbits were immunized subcutaneously (s.c.) with 0.5 ml of each fraction or whole MPV emulsified in 0.5 ml of Freund complete adjuvant once weekly for 3 weeks. Rabbits were bled by cardiac puncture 0, 7, 14, and 27 days post initial immunization. Sera were heat inactivated at 56°C for 30 min and stored at −20°C until used.

(ii) Rhesus monkeys—immunization and challenge. Two of the three rhesus monkeys, each weighing 4.5 kg, were "vaccinated" with the saline-soluble XP fraction, and the third monkey, unvaccinated, was used as a control. The two monkeys were each vaccinated four times, with a 1-week waiting period after each vaccination. They were initially immunized s.c. with a mixture of equivalent (0.5 ml) amounts of emulsified XP fraction and Freund complete adjuvant followed by three weekly s.c. injections of XP fraction emulsified in Freund incomplete adjuvant.

Two weeks after the fourth inoculation, the two vaccinated monkeys and the control monkey were each challenged s.c. with 0.8 ml of live Utrecht strain of MPV, grown on chicken chorioallantoic membranes, containing $8 \times 10^{4.6}$ pock-forming units. On the sixth day after the challenge, the three monkeys were again challenged with $8 \times 10^{6.5}$ pock-forming units of live Utrecht strain of MPV. The second challenge was given to assure "adequate" dosage. The three monkeys were observed daily for 42 days after the first challenge.

After the first vaccination, blood specimens were collected from the femoral veins of the three monkeys on day 0, and additional blood specimens were collected from only the two vaccinated monkeys on days 3, 7, 10, 17, 21, and 28. After the first challenge, all three monkeys were bled on days 3, 6, 9, 16, 29, and 42. Each specimen was assayed for antibodies by hemagglutination-inhibition (HI), complement fixation (CF), and VN tests. The blood specimens from all three monkeys collected on days 3 and 6 after the first challenge inoculation were mixed with ethylenediaminetetraacetate and centrifuged. The buffy coats obtained from the centrifuged blood specimens were inoculated onto chorioallantoic membranes to test for the presence of MPV. Scabs of lesions found on the unvaccinated monkey on day 9 after the first challenge inoculation were also tested for the presence of MPV.

Plaque reduction neutralization assay. Stock MPV was diluted to equal 50 to 100 plaque-forming units (PFU)/0.1 ml. Serial twofold dilutions of heat-inactivated rabbit sera, initially diluted 1:5, were prepared in MEM medium. Equivolumes (0.5 ml) of virus and sera were incubated at room temperature for 60 min. Four 60-mm petri dishes containing confluent LLC-MK_2 cells were inoculated with 0.2-ml virus-serum mixtures and adsorbed for 60 min at 37°C with rocking. Cells were fed with MEM plus 2% FBS and incubated for 48 h. Cells were fixed and stained as described previously, and plaques were enumerated at 48 h postinfection (as a control, diluted virus was simultaneously incubated and assayed with the virus-serum mixtures). Titers are expressed as that dilution of serum which reduced plaque formation by 50%.

Hemagglutination and HI tests. Hemagglutination and HI tests were performed according to the method of Hierholzer and Suggs (3) and Hierholzer et al. (4).

CF test. CF tests were performed according to the method of Palmer and Casey (11).

RESULTS

Fractionation of MPV. Table 1 summarizes the results of the initial evaluation of the immunogenicity of soluble and insoluble X-press

TABLE 1. *Fractionation of purified MPV by mechanical disruption: analysis of soluble and insoluble fractions for eliciting VN antibody*

Virion fraction	Surviving virus infectivity after treatment[a]	Rabbits immunized[b]	VN response to MPV[c]	CF response to FBS
Untreated	5×10^5	R-29	≥1:160	NT[d]
Disrupted by X-press				
Soluble[e]	0	R-120	1:160	<1:2
Fraction		R-122	1:20	<1:2
Insoluble[f]	0	R-139	<1:5	NT
Fraction		R-140	<1:5	NT

[a] Tested on LLC-MK_2 cells.

[b] R-29 was scarified with 5×10^5 PFU of MPV per ml and bled 45 days postinfection. All other animals were immunized with approximately 10 mg of virus protein per ml once a week for 3 weeks and exsanguinated after the 4th week.

[c] Determined by 50% reduction in plaque-forming units.

[d] NT, Not tested.

[e] Virion fraction soluble in saline after mechanical disruption by X-press.

[f] Virion fraction insoluble in saline after mechanical disruption.

fractions from purified MPV. Rabbits immunized with the soluble fraction produced VN antibody titers of 1:160 and 1:20, whereas rabbits immunized with the insoluble fraction elicited VN titers of <1:5. No response to FBS was detected in the rabbits immunized with the soluble fraction. No detectable infectious virus was recovered from either fraction.

Hemagglutination activity of the X-press fractions. The saline-soluble XP fraction and the saline-insoluble SUM fraction before treatment with the mixture of sodium dodecyl sulfate, urea, and 2-mercaptoethanol did not hemagglutinate sensitive chicken erythrocytes.

The SUM fraction treated with the mixture of sodium dodecyl sulfate, urea, and 2-mercaptoethanol was not determined for hemagglutination beyond the observation that the fraction hemolyzed the erythrocytes.

Evaluation of vaccine potential of MPV-soluble fraction. Table 2 summarizes the results of the serological responses, virus isolations, and clinical observations of XP-immunized rhesus monkeys subsequently challenged with infectious MPV. The three monkeys used in the investigation had no known previous clinical exposure to MPV and were free of VN, HI, and CF antibodies to MPV. Monkeys 1 and 2 produced VN antibody titers of 1:1,200 by day 28 postimmunization. In addition to the VN antibody, the two immunized monkeys produced HI titers of 1:80 and 1:160, respectively. Furthermore, monkey 1 produced a CF titer of 1:16, and the response of monkey 2 was obscured by anticomplementary activity. The two immunized monkeys remained clinically free of monkey pox disease, whereas the unimmunized control monkey became ill 7 days after challenge with typical monkey pox disease with lesions that progressed through the maculopapular, the vesicular, and the scabbing stages; it recovered fully from the disease in 3 weeks (Fig. 1). Viremia was prevented in the immunized monkeys in that no virus was recovered from blood samples collected 3 and 6 days postchallenge, whereas virus was recovered from the control monkey 3 days postchallenge but was not recovered by day 6. Furthermore, virus was recovered from the control monkey's skin lesions collected 9 days postchallenge. Thus, these results indicated adequate virus challenge. Whereas the XP fraction elicited an HI response in immunized monkeys, this fraction contained no hemagglutination activity.

DISCUSSION

An X-press has been used successfully to mechanically disrupt and release structural proteins from vaccinia virus (8) and Yaba tumor poxvirus (10). At least four structural proteins

TABLE 2. *Evaluation of mechanically disrupted MPV vaccine in rhesus monkeys*

Day tested	Titer in: Vaccinated monkey 1[a]				Vaccinated monkey 2[a]				Control monkey 3[b]			
	VN	CF	HI	Viremia	VN	CF	HI	Viremia	VN	CF	HI	Viremia
Prechallenge												
0	<4	<8	<5		<4	<8	5		<4	<8	<5	
3	<4	<8	<5		<4	<8	5		NT[c]	NT	NT	
7	<4	<8	<5		<4	<8	5		NT	NT	NT	
10	<4	<8	20		<4	≤16[d]	10		NT	NT	NT	
17	<4	<8	40		<4	≤16[d]	40		NT	NT	NT	
21	<4	<8	40		32	≤16[d]	80		NT	NT	NT	
28	1,200	16	80		1,200	≤16[d]	160		NT	NT	NT	
Postchallenge[e]												
3	1,400	8	80	–[f]	1,400	64	160	–	<4	<8	<5	+[g]
6	8,000	32	160	–	2,800	128	320	–	<4	<8	5	–
9	1,600	32	160		10,000	256	320	–	14	<8	160	
16	500	128	80		5,700	≥256	320		20	256	640	
29	2,200	128	40		320	256	80		200	256	320	
42	1,600	≥256	80		9,000	≥256	80		1,024	≥256	320	

[a] Vaccinated four times with 5 mg (total of 20 mg) of soluble fraction of monkey pox prepared by mechanical disruption (see text).
[b] Nonvaccinated monkey.
[c] NT, Not tested.
[d] Anti-complimentary at 1:8.
[e] Monkeys were challenged with $8 \times 10^{4.6}$ pock-forming units of MPV (Utrecht isolate).
[f] Negative virus recovery.
[g] Positive virus recovery.

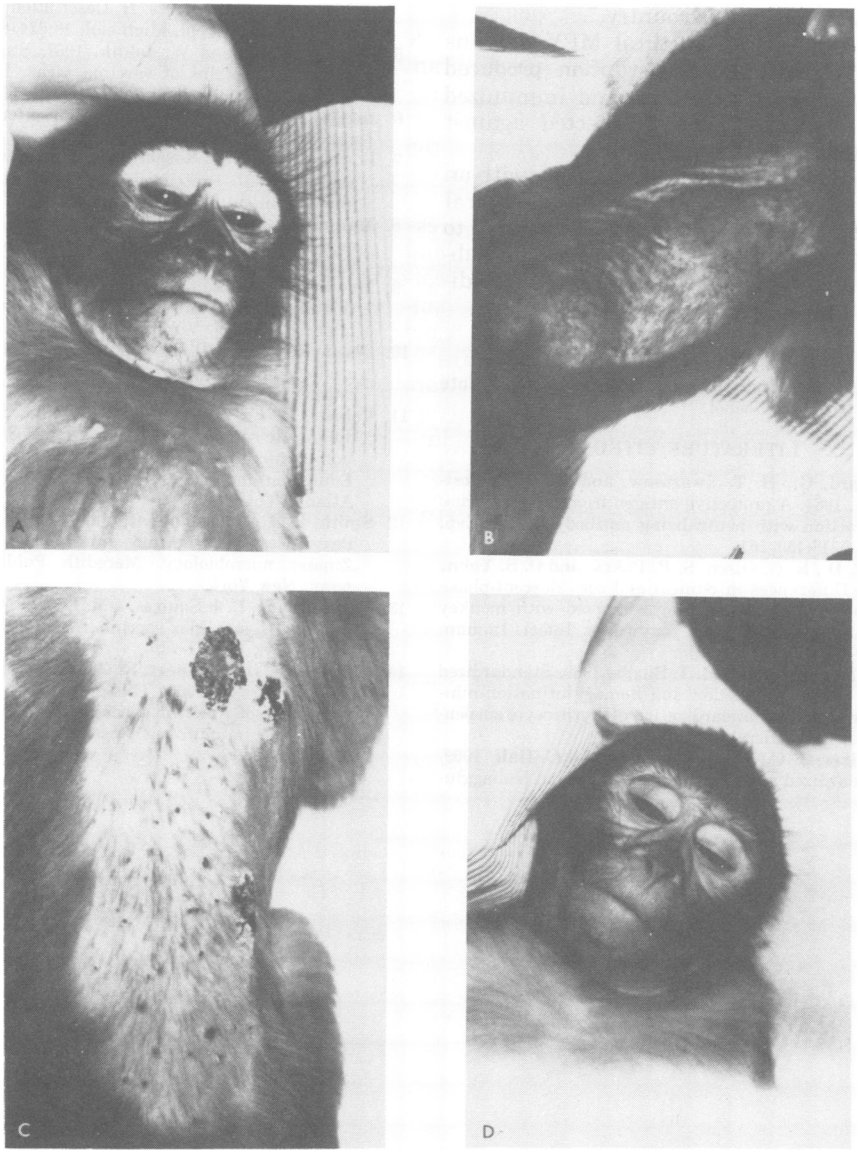

FIG. 1. *Development of pox in rhesus monkeys after challenge with virulent MPV. The nonimmunized monkey developed facial (A) and abdominal vesicles 9 days postinfection (B). Scabbing developed by 16 days postinfection (C). Monkeys immunized with the soluble fraction of MPV developed no pox skin lesions (D).*

were released from the mechanically disrupted Yaba tumor virus, which elicited the production of Yaba VN antibody (10). We showed in this study that mechanically disrupted MPV also released structural proteins that elicited the production of HI, CF, or VN antibodies in rabbits and rhesus monkeys. The rabbits were immunized with the soluble XP and insoluble SUM fractions prepared from purified MPV, whereas the rhesus monkeys were immunized only with the soluble XP fraction prepared from concentrated washed virus pellets. In both cases, all infectious virus was destroyed.

To attain a safe vaccine, many investigators tested the use of smallpox vaccine composed of inactivated vaccinia virus, but most concluded that the inactivated smallpox vaccines were dubiously effective. Turner et al. (13) compared antibody responses of vaccines inactivated by six methods and concluded that "the efficacy of inactivated vaccines in preventing subsequent vaccination accidents in man has yet to be ade-

quately assessed in this country."

In this study the structural MPV proteins released by mechanical disruption produced VN, HI, and CF antibodies, and immunized monkeys were completely protected against MPV-induced disease.

The results of this preliminary study with an MPV model suggest that solubilization of viral proteins by mechanical disruption may lead to the development of an efficacious virion subunit vaccinia vaccine for use in high-risk individuals.

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LITERATURE CITED

1. **Appleyard, G., H. T. Zwartouw, and J. C. N. Westwood.** 1964. A protective antigen from the pox-virus. I. Reaction with neutralizing antibody. Br. J. Exp. Pathol. 45:150-161.
2. **Hall, R. D., R. G. Olsen, S. P. Pakes, and D. S. Yohn.** 1973. Differences in clinical and convalescent-phase antibody of rhesus monkeys infected with monkey pox, tanapox and Yaba poxviruses. Infect. Immun. 7:539-546.
3. **Hierholzer, J. C., and M. T. Suggs.** 1969. Standardized viral hemagglutination and hemagglutination-inhibition tests. I. Standardization of erythrocyte suspensions. Appl. Microbiol. 18:816-823.
4. **Hierholzer, J. C., M. T. Suggs, and E. C. Hall.** 1969. Standardized viral hemagglutination and hemagglutination-inhibition tests. II. Description and statistical evaluation. Appl. Microbiol. 18:824-833.
5. **Holowczak, J., and W. Joklik.** 1967. Studies on the structural proteins of vaccinia virus. I. Structural proteins of virions and cores. Virology 33:717-725.
6. **Joklik, W. K.** 1962. The purification of four strains of poxvirus. Virology 18:9-18.
7. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
8. **Marquardt, J., W. E. Holm, and E. Lycke.** 1965. Immunoprecipitation factors of vaccinia virus. Virology 27:170-178.
9. **Munyon, W., J. Mann, and J. T. Grace, Jr.** 1970. Protection of vaccinia from heat inactivation by nucleotide triphosphates. J. Virol. 5:32-38.
10. **Olsen, R. G., and D. S. Yohn.** 1970. Immunodiffusion analysis of Yaba poxvirus structural and associated antigens. J. Virol. 5:212-220.
11. **Palmer, D. F., and H. L. Casey.** 1974. Standardized diagnostic complement fixation method and adaptation to micro test, p. 56. U.S. Department of Health, Education and Welfare, Center for Disease Control, Atlanta, Ga.
12. **Smith, D. T., N. F. Conant, and J. R. Overman.** 1964. Poxviruses of man and animals, p. 903-907. *In* Zinsser microbiology. Meredith Publishing Company, New York.
13. **Turner, G. S., E. J. Squires, and H. G. S. Murray.** 1970. Inactivated smallpox vaccine. A comparison of methods. J. Hyg. 68:197-210.
14. **Warnaar, S. O., E. Knegt, R. Charles, R. Schilperoort, and W. Grose.** 1965. The preparation by alkaline treatment of a serum blocking antigen of vaccinia virus with immunizing potency. Arch. Virusforsch. 17:577-584.