Effect of Proteolytic Enzymes on the Hemagglutinating Property of the Parvoviruses, H-1, H-3, and RV[1] (36411)

JOHN R. KONGSVIK, AND HELENE W. TOOLAN

Putnam Memorial Hospital Institute for Mecical Research, Bennington, Vermont 05201

The H-viruses, members of the Parvovirus group, agglutinate various types of erythrocytes with such specificity that they can often be distinguished from one another by the type of red cell with which they react (1). Agglutination is an integral property of the virus particles (2, 3), which form a bridge between two or more erythrocytes (4). While Greene (2) noted that the hemagglutinating (HA) property of H-1 virus was resistant to a wide range of pH and heat, Castro *et al.* (5) have recently reported that the HA ability of some unpurified Parvovirus (H-1, RV and X14) preparations could be chemically altered. No effort was made by these authors to determine whether the reagents they employed acted upon viral or red cell receptors. The present study was undertaken to determine whether the HA ability of highly purified H-viruses could be modified chemically; attempts were also made to delineate the site of action of the compounds used.

Materials and Methods. Viruses. Cell culture and virus propagation methods have been described elsewhere (6). In this study, hamster embryo cultures were used for propagating H-1 and H-3, while RV was grown in rat embryo cultures. Virus was purified by a recently-developed detergent method, utilizing sucrose velocity sedimentation and isopycnic banding in CsCl (S. L. Rhode, manuscript in preparation). When examined under the electron microscope, purified and concentrated virus obtained by this method appeared clean, intact and in solid sheets, while the HA titer of the preparations had an average end point dilution of 1:81,920 to 1:163,840. All virus and reagent dilutions were done with phosphate-buffered saline (PBS) (0.01 *M* PO_4–0.15 *M* NaCl, pH 7.2). Crude H-1 was obtained by ultrafiltration of media from infected cultures, followed by sedimentation of the virus at 40,000 rpm for 2 hr.

Hemagglutination assay. Treatment of viruses with reagents. Equal volumes (0.5 ml) of virus (50 μl of purified virus was brought to 2 ml with PBS, *i.e.*, at 1:40 dilution) and reagents were mixed and incubated for the specified times at 37°. Untreated virus was diluted and incubated under the same conditions and for the same times. After incubation, 8 ml of PBS was added to each tube to prevent collapse of the tubes during subsequent centrifugation at 40,000 for 2 hr. Each series of tests was spun in the same rotor to rule out variations from run to run. The supernatant fluids (9 ml) were tested directly for HA, while the virus pellets were resuspended to the original volume of 1 ml with PBS before assay. In one case, after incubation, the virus and reagents were dialyzed for 48 hr at 5° against 0.01 *M* phosphate buffer, pH 7.2. Hemagglutination assays were performed in plastic trays using 0.8 ml of treated or control virus and 0.4% guinea pig red cells. After standing overnight at 5°, the titers were read as the highest dilution at which HA was complete. Reagents used were *p*-chloromercuribenzoate (PCMB, Sigma); papain, trypsin, pepsin and neuraminidase (Worthington); pronase (Sigma) and mercaptoethanol (Eastman). Infectivity of control and enzyme-treated viruses after sedimentation was tested in newborn hamsters.

Treatment of red cells with papain. A 0.2 ml volume of washed and packed guinea pig red blood cells (RBC) was suspended in 5

[1] This investigation was supported by National Cancer Institute, N.I.H., U.S. Public Health Service Grant CA-07826-07, and by a generous gift from the Given Foundation.

TABLE I. Agglutination of Guinea Pig Red Cells by Purified H-Viruses.

Virus and treatments	Exps. A[a] Pellet	Exps. A[a] Supernatant	Exp. B[b]
H-1 + PBS (control)	8[c]	8	12
H-1 + PCMB (0.005 *M*)	5	6	10
H-1 + PCMB + ME[d]	7	5	10
H-1 + papain (20 U/ml)	12	8	11
H-3 + PBS (control)	5	5	9
H-3 + PCMB	3	2	8
H-3 + PCMB + ME	5	4	8
H-3 + papain	10	1	6
RV + PBS (control)	3	3	8
RV + PCMB	3	2	7
RV + PCMB + ME	3	3	7
RV + papain	8	1	8

[a] Viruses and reagents were incubated for 60 min at 37° and separated by centrifugation at 40,000 rpm for 2 hr. The pellets and supernatants were then tested for HA. Three additional experiments gave similar results.

[b] Viruses and reagents were incubated for 60 min at 37°, dialyzed for 2 days at 5° and tested for HA. One experiment only.

[c] Titers are expressed as that dilution at which hemagglutination was complete. 1=1:10; 2=1:20; 3=1:40; 4=1:80; 5=1:160; 6=1:320; 7=1:640; 8=1280, etc.

[d] Reversal of PCMB inhibition affected by 0.1% mercaptoethanol in PBS used as a diluent for HA (A) or by dialysis of sample against 0.1% mercaptoethanol in PBS for 2 days at 5° (B).

ml of PBS (control) or 5 ml of papain (final concentration 20 U/ml). After incubation at 37°, control and treated RBC were pelleted, washed once with PBS and diluted to 0.4% before assay.

Polyacrylamide gel electrophoresis. Gels were prepared by the method of Summers *et al.* (7). Enzyme treated and control virus were solubilized with an equal volume of 4% sodium dodecyl sulfate (SDS) and 2% mercaptoethanol in 0.01 *M* phosphate buffer by heating for 1 min at 100°. Virus polypeptides were layered on 7.5% acrylamide gels and run for 3–4 hr at 5 mA/tube. The buffer used in the tray was 0.1 *M* phosphate containing 0.1% SDS. Gels were fixed in 10% trichloroacetic acid (TCA) overnight and stained with 0.1% coomassie blue in 50% methanol containing 7.5% acetic acid (8). Destaining was done by soaking the gels in 7% acetic acid containing 20% ethanol.

Results. Incubation of purified viruses with the sulfhydryl reagent, PCMB, depressed the HA titer of both the pellet and supernatant fractions of H-1 and H-3, while there was little effect on RV (Table I, Exps. A). Partial reversal of PCMB inhibition by mercaptoethanol (ME) indicated that SH groups are essential to some aspect of the HA reaction, but did not reveal whether viral or erythrocyte receptors were affected. The same initial concentration of PCMB, however, diluted out during HA titrations, lysed the RBC to a titer of 5. At the concentration used (0.005 *M*), therefore, most of the PCMB added to the virus preparations remained bound to either the pelleted, intact virus or the supernatant components and could be partially removed by mercaptoethanol treatment.

Treatment of virus with the proteolytic enzyme, papain, increased the HA titer of pelleted H-1, H-3 and RV (Table I Exps. A), by 50%, 100%, and 167%, respectively. Unpelleted H-1 virus capsomeres or soluble components, on the other hand, were not affected by the papain treatment though in the case of H-3 and RV, there was a decrease in HA of the soluble or minute particulate material. This latter finding would be expected if the small capsomeres resulting from spontaneous degradation of H-3 and RV are digested by papain, while the capsomeres of H-1 and the pellets of all the intact virions are not. Other enzymes tested but not included in the table were pepsin, trypsin, protease and neuraminidase. None affected HA of the intact virus, except neuraminidase, which reduced the HA of the pelleted virus from 1 to 2 logs and completely abolished HA of the supernatant fractions. This substantiates the known receptor-destroying effect of the neuraminidase, most of which remained in the supernatant. The permanence of the PCMB and papain effect on virus receptors was also evaluated by dialysis. Dialysis of the virus–reagent mixture after incubation resulted in the absence of mercaptoethanol reversal of PCMB inhibition of all three viruses, with a concomitant decrease in HA of papain-treated H-1 and H-3 (Ta-

TABLE II. Effect of Length of Incubation with Papain on HA of Viruses.

Virus and treatment	Time (min)	Pellet	Supernatant
H-1 + PBS (control)	60[a]	10	8
H-1 + papain	30	10	8
H-1 + papain	60	11	5
H-1 + papain	120	10	8
H-1 + papain	180	10	8
H-3 + PBS (control)	60	6	5
H-3 + papain	30	8	2
H-3 + papain	60	7	5
H-3 + papain	120	7	4
H-3 + papain	180	8	4
RV + PBS (control)	60	3	5
RV + papain	30	7	2
RV + papain	60	6	3
RV + papain	120	6	4
RV + papain	180	6	3

[a] Viruses were incubated with papain (20 U/ml) at 37° for the times indicated. Virus was then separated from papain by centrifugation at 40,000 rpm for 2 hr. Pellets and supernatants were then tested for HA activity. See legend of Table I for explanation of HA titers.

ble IB). Some PCMB is therefore irreversibly bound to the H-viruses and affects their HA activity. Either the papain-induced modifications of virus receptors which occurred during incubation are overcome during dialysis, or prolonged dialysis of papain at 5° is detrimental to any action it may have on red cells during the HA assay.

The striking increase in the HA titers of papain-treated virus prompted further study into possible mechanism(s) of action. If the effect is primarily on the virus, then the increase in HA could be expected to vary as a function of time of incubation with papain. The results of incubating H-1, H-3 and RV at 37° with papain for 30, 60, 120 and 180 min before sedimentation are shown in Table II. It is evident that after 30 min incubation with papain, no further increase occurred in HA titer. Furthermore, when papain was added to the virus preparations immediately before HA assay no increase in titer above that of the controls occurred. It is possible, therefore, that papain is removing a covalently-bound contaminant from the purified virus surface, thereby exposing more receptor sites. The fact that no change was seen in the characteristic electrophoretic pattern of H-1 capsid components (9) after papain treatment would tend to substantiate this. When a crude H-1 preparation was substituted for the purified virus and incubated with papain under the same conditions, it took longer for papain to affect the HA titer of the pelleted virus (Table III). Presumably, in this case at least, the main effect of papain was to release virus from associated cellular debris.

The effect of treating the RBC with papain prior to a HA test with H-1, was also studied. Red cells with and without papain were incubated at 37° for 30, 60, 180, 240 and 360 min before HA assay. After washing the RBC with PBS, H-1 was added in the usual manner for an HA test. The results are shown in Table IV. It appears that papain-induced modification of the RBC is almost complete after 30 min. At that time the treated red cells alone gave a 2+ hemagglutination while the addition of virus showed a titer of 1:10,240 (the final dilution with ++++ reading) as compared with 1:1280 for the control. By 60 min the treated RBC alone showed ++++ HA and all readings for the virus became meaningless. This finding indicated that the site of action of papain was primarily on the red cell. The addition of papain by itself to the assay wells immediately before addition of red cells gave a titer of 10. The modification, therefore, can occur at once and at 5°.

TABLE III. Agglutination of Guinea Pig Red Cells by Unpurified H-1.[a]

Virus and treatments	Time (min)	Pellet	Super
H-1 + PBS (control)	60	6[b]	1
H-1 + PCMB (0.005 *M*)	60	3	2
H-1 + Papain (20 U/ml)	30	5	0
H-1 + Papain	60	5	1
H-1 + Papain	120	7	0
H-1 + Pepsin (250 μg/ml)	60	4	1
H-1 + Trypsin (250 μg/ml)	60	6	1

[a] Unpurified H-1 was incubated for the specified times at 37°. After centrifugation at 40,000 rpm for 2 hr HA assays were performed as described in Methods.

[b] See legend of Table I for explanation of HA titers.

TABLE IV. Effect of Time of Incubation with Papain on the Agglutinability of Red Cells by H-1.[a]

Treatment	Time (min)	HA
H-1 + RBC control	60	8
RBC + PBS	—	0
RBC + papain + PBS	39	++[b]
RBC + papain + H-1	30	11
RBC + papain + PBS	60	++++[c]
RBC + papain + H-1	60	++++(24)[d]
RBC + papain + PBS	180	++++
RBC + papain + H-1	180	++++(24)
RBC + papain + H-1	240	++++(24)
RBC + papain + H-1	360	++++(24)

[a] Guinea pig erythrocytes were incubated with papain (20 U/ml) at 37° for the indicated times, washed and then compared with untreated, but incubated, control erythrocytes for agglutinability with PBS or H-1 virus. (See legend of Table I for explanation of HA titers.)

[b] Degree of agglutination indicated by number of plus signs (*i.e.*, ++++ = complete HA; ++ = incomplete).

[c] Indicates that HA pattern in all eight wells used was indistinguishable from that obtained when virus acts as the HA agent.

[d] Virus agglutinated treated cells in all 24 wells used.

Discussion. The sensitivity of the HA activity of purified H-viruses to PCMB suggests that virus-bound SH groups are somehow involved in the agglutination reaction. Similar, but more pronounced inhibition by PCMB was reported by Castro *et al.* (5) for three unpurified H-viruses. In contrast to the finding of these authors, however, we observed that most of the PCMB inhibition could be reversed by mercaptoethanol.

Treatment of purified H-1, H-3 and RV with pepsin, trypsin and pronase had no effect on their HA activity, while papain produced a significant increase in the HA titers of all three viruses. The latter effect was apparently limited to virus HA activity only as all the virus pellets, whether control or treated with any of the enzymes including papain, were infectious to the same degree (an equal LD_{50}) for each virus when injected into newborn hamsters. The resistance of these small DNA viruses to degradation by proteolytic enzymes is characteristic (10), and was also substantiated in this study by SDS–acrylamide gel electrophoresis of papain-treated virus which had a pattern similar to that of the control virus. It seems likely that papain affects the virus by removing a tightly-bound HA-inhibiting contaminant. The demonstration that papain enhances both the HA activity of H-viruses and the agglutinability of guinea pig red cells may be unique to the Parvoviruses. It has been noted, for example, that trypsin increases the HA of reovirus 1, but not that of reo 2 or 3, while it abolishes the receptors of human red cells to all three virus types (11).

Papain-induced enhancement of unpurified H-viruses as described by Castro *et al.* (5) was explained as the release of virus from cellular debris. No attempt was made, however, to study the effect of papain on the red cell receptors. Based on the studies reported here, it seems likely that papain, in addition to removing convalently-bound contaminants from the purified virus, may sediment in small quantities with the virus and act on the red cell surface in subsequent HA tests. Papain is known to enhance the agglutinability of red cells (12, 13), possibly by reducing the net surface charge density of the erythrocytes (14). Since the RBC surface has a net negative charge due to sialic acid residues (15), the esterase activity of papain could reduce the electrostatic repulsion between adjacent red cells, thus permitting closer cell–cell or cell–virus contact. In the case of H-1 which is negatively charged (9) the virus could more readily agglutinate papain-treated red cells if the negative surface charge on the latter was reduced.

It appears, therefore, from these studies that papain has a dual effect on the parvovirus HA property; one on the virus surface which exposes receptors masked by bound contaminants and another on the red cell surface probably involving a surface charge reduction. Both effects, in combination, produce an additive result; they are probably related to the fact that the action of papain is nonspecific.

Summary. Three purified Parvoviruses (H-1, H-3 and RV), treated with various proteolytic enzymes, showed chemically-induced

changes in their hemagglutination pattern only after exposure to papain which enhanced the values found. This increase was probably due to a dual effect of the papain: one on the virus itself by removing bound contaminants and, two, on the red cell membrane, probably by reducing the negative charge. Although the HA titer of virus was altered by papain treatment, no change in the SDS–acrylamide electrophoretic pattern of the capsid proteins or in infectivity was observed.

The authors thank Miss Barbara Madden for technical assistance.

1. Toolan, H. W., Proc. Soc. Exp. Biol. Med. **124**, 144 (1967).

2. Greene, E. L., Proc. Soc. Exp. Biol. Med. **118**, 973 (1965).

3. Payne, F. E., Beals, T. F., and Preston, R. E., Virology **23**, 109 (1964).

4. Portella, O. B., Arch. Virusforsch. **14**, 277 (1963).

5. Castro, A., Nicoletti, G., and Cocuzza, G., Arch. Virusforsch. **34**, 261 (1971).

6. Al-Lami, F., Ledinko, N., and Toolan, H. W., J. Gen. Virol. **5**, 485 (1969).

7. Summers, D. F., Maizel, J. V., Jr., and Darnell, J. E., Jr., Proc. Nat. Acad. Sci. U.S.A. **54**, 505 (1965).

8. Maizel, J. V., Jr., Summers, D. E., Jr., and Scharff, M. D., J. Cell. Physiol. **76**, 273 (1970).

9. Kongsvik, J. R., and Toolan, H. W., Proc. Soc. Exp. Biol. Med. **139**, 1202 (1972).

10. Greene, E. L., and Karasaki, S., Proc. Soc. Exp. Biol. Med. **119**, 918 (1965).

11. Lerner, A. M., Cherry, J. D., and Finland, M., Virology **19**, 58 (1965).

12. Leikola, J., Int. Arch. Allergy **33**, 112 (1968).

13. Leikola, J., and Pasanen, V. J., Int. Arch. Allergy **39**, 352 (1970).

14. Pollack, W., and Hager, H. J., Proc. 10th Congr. Int. Soc. Blood Transf. 802 (1965).

15. Eylar, E. H., Madoff, M. A., Brady, O. V., and Oncley, J. L., J. Biol. Chem. **237**, 1992 (1962).

Received Jan. 17, 1972. P.S.E.B.M., 1972, Vol. 140.