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NOTES

Hemolysis by Liposomes Containing Influenza Virus Hemagglutinins

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Liposomes containing influenza virus hemagglutinin were reassembled from envelopes solubilized with Nonidet P-40 and were shown to induce hemolysis and cell fusion at low pH.

There is suggestive evidence that influenza virus hemagglutinin is responsible for virus-induced hemolysis: (i) influenza virus with the uncleavage type of hemagglutinin (HA_0) did not induce hemolysis or cell fusion (4, 6, 14), whereas virus with the cleavage type of hemagglutinin ($HA_1 + HA_2$) induced both (4, 6, 14); (ii) the optimum pH for hemolysis by recombinant influenza viruses is the same as that of the parent virus from which the hemagglutinin is derived (12); and (iii) virus-induced hemolysis is not dependent on the neuraminidase activity of the virus since it is not affected by heat inactivation of neuraminidase activity or depletion of neuraminidase by trypsin treatment (4, 16). However, no direct evidence that the hemagglutinin is involved in virus-induced hemolysis has been reported. As described here, we reassembled envelope particles from Nonidet P-40 (NP-40)–solubilized influenza virus envelopes by removal of the detergent by dialysis and demonstrated that the only virus constituent glycoprotein of these particles was the hemagglutinin and that the particles induced hemolysis and cell fusion.

Influenza virus of strain PR8 was propagated in the chorioallantoic cavity and purified by differential centrifugation followed by velocity sedimentation in a sucrose gradient in 0.15 M NaCl–0.015 M sodium citrate (3). The purified virions were suspended in 0.005 M Tris buffer (pH 7.4), solubilized with 0.25% NP-40 at room temperature for 15 min, and centrifuged at 100,000 × *g* for 40 min. The supernatant was dialyzed for 3 days in Spectrum membrane tubing no. 2 (Spectrapor Medical Industries, Inc., Los Angeles, Calif.) against phosphate-buffered saline containing 1 mM $MgCl_2$ (5), with SM2 beads (Bio-Rad Laboratories, Richmond, Calif.) outside the tubing (17). The dialysate was centrifuged at 100,000 × *g* for 40 min, and the pellet was suspended in phosphate-buffered saline and used as reassembled envelope particles (REP).

The viral dose was expressed in hemagglutination units (HAU). HAU were measured by the pattern method on a microscale (2). Hemolysis was measured spectrophotometrically as the increase in absorbance at 540 nm owing to the liberation of hemoglobin after incubation of 1 ml of 1% human erythrocytes (type O) with a small volume of viral sample at pH 5.3 (0.05 M acetate-buffered saline) at 37°C for 60 min followed by the addition of 1 ml of phosphate-buffered saline (pH 7.2) to stop the reaction. Values were expressed as percentages of the total lysis achieved with 0.045 M NH_4OH. The cell fusion experiment was done as follows: a 100-μl sample of 1% human erythrocytes in phosphate-buffered saline was mixed with 25 μl of REP (1,000 HAU) in the cold for 15 min, and then the medium was exchanged for 200 μl of the fusion medium (acetate-buffered saline containing 0.2% bovine serum albumin [Difco Laboratories, Detroit, Mich.], pH 5.3). The reaction mixture was incubated at 37°C for 30 min and observed by light microscopy. Neuraminidase activity was measured by the method of Aminoff (1). Protein was determined by the method of Lowry et al. (13). Gel electrophoresis was done in 10% gel by the procedure of Laemmli (9), and bands of proteins were stained with Coomassie blue.

Figure 1 compares the dose-response curves of REP and of PR8 virions for hemolysis. At the same dose of HAU, the hemolytic activity of REP appeared to be similar to that of virions, or a little higher.

Figure 2 shows the dose-response curves of

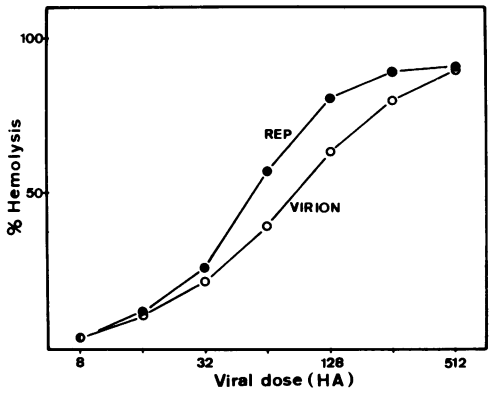

FIG. 1. Dose-response curves of REP (●) and of PR8 virions (○) for hemolysis. Portions (100 μl) of twofold serial dilutions of REP and of PR8 virions were mixed with 1 ml of 1% human erythrocytes at pH 5.3 for 15 min in the cold and were incubated at 37°C as described in the text. HAU per milligram of protein were measured as 8×10^4 (virions) and 30×10^4 (REP).

REP and virions for neuraminidase activity. The activity of REP was almost negligible with doses of up to 1,600 HAU, whereas that of virions increased with the virion dose. Since NP-40 treatment was found not to affect neuraminidase activity, the lack of the activity suggests a negligible association of the REP preparation with neuraminidase protein. Figure 3 shows the patterns of virions and REP in polyacrylamide gel electrophoresis. REP was shown to contain

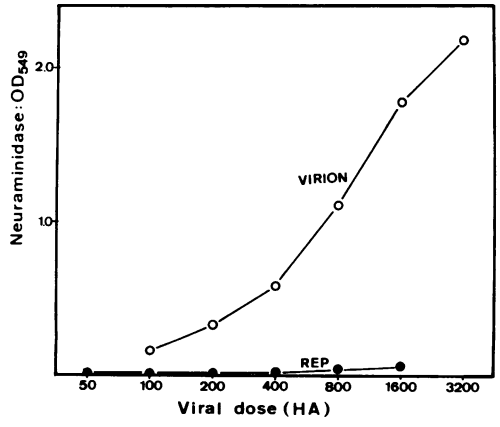

FIG. 2. Dose-response curves of REP (●) and of PR8 virions (○) for neuraminidase activity, measured by optical density at 549 nm (OD_{549}). Portions (25 μl) of twofold serial dilutions of REP and of PR8 virions were incubated with 100 μl of Fetuin (GIBCO Laboratories Grand Island, N.Y., Spiro method; 10 mg/ml) at pH 5.5 at 37°C for 30 min. Liberated sialic acid was estimated by the method of Aminoff (1).

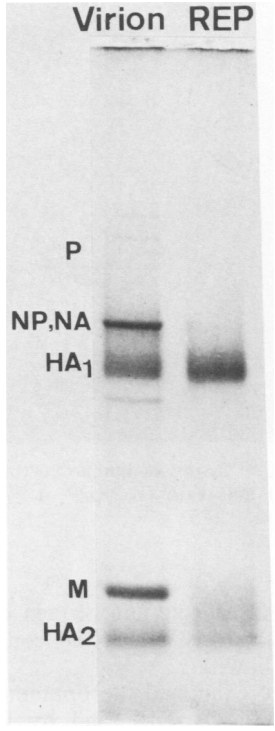

FIG. 3. Gel electrophoresis of REP and of PR8 virions. The nomenclature of virus polypeptides is according to Kilbourne et al. (8) and Lamb and Choppin (10). The broad band of material moving a little more slowly than the HA_2 band was not exactly identified. Amounts of proteins applied were 25 and 15 μg from virions and REP, respectively.

the HA_1 and HA_2 polypeptides, which were the only identified proteins, and also a broad band of material moving a little more slowly than HA_2. This material might be the HA_2 polypeptide with conformational changes produced during NP-40 treatment followed by sodium dodecyl sulfate–mercaptoethanol dissociation. This interpretation was supported by the finding that, although the density of the HA_1 band of REP was higher than that of virions, the density of the HA_2 band of REP was lower than that of virions; therefore, some HA_2 of REP should be found elsewhere to compensate for the reduced amounts of HA_2 of the right mobility. However, the possibility that this material was the proteolytic product of the M protein (11) cannot be eliminated at present. The neuraminidase band was not detected in the REP. Since the minimum 0.5 μg of bovine serum albumin was resolved in the gel electrophoresis system used, the possible content of the neuraminidase contaminating the REP preparation was less than 3% (0.5 μg/15 μg of protein applied). Neuraminidase was negligible in the REP because treatment with 0.25% NP-40 solu-

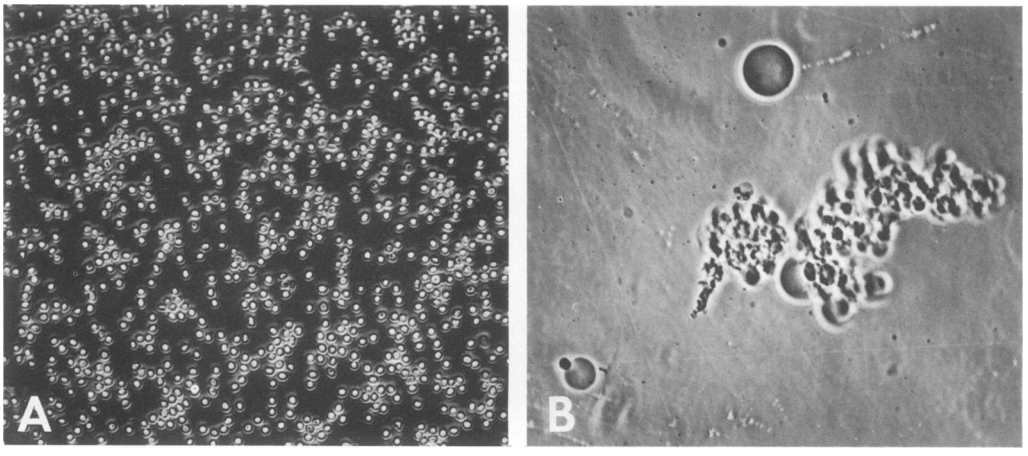

FIG. 4. Cell fusion of human erythrocytes with the REP of PR8 virus. The erythrocytes were incubated without (A) and with (B) REP at 37°C for 30 min. Experimental conditions are described in the text. Magnification, ×175.

bilized the hemagglutinin protein of PR8 virus almost exclusively under the conditions used (data not shown).

Next, we examined the optimum pH values for hemolysis by REP and by PR8 virions. Almost the same optimum pH values (5.2 to 5.4) were found for both REP and virions, although the optimum for REP tended to be slightly widened and shifted towards neutrality.

Figure 4 shows cell fusion of human erythrocytes by REP. Under the conditions used, a lot of cell aggregates were formed after incubation for 30 min, making it difficult to quantitate the cell fusion. As shown in Fig. 4, giant erythrocytes were efficiently induced with REP.

Figure 5 is an electron micrograph of the REP, which consisted of circular and linear membramous particles covered with spikes 10 nm long.

The present results clearly demonstrate that membramous particles containing predominantly influenza virus hemagglutinin induce hemolysis and cell fusion at low pH. This finding is consistent with the recently published finding that the hemagglutinin of influenza virus expressed from a cloned gene induces cell fusion (18). Furthermore, it is consistent with the hy-

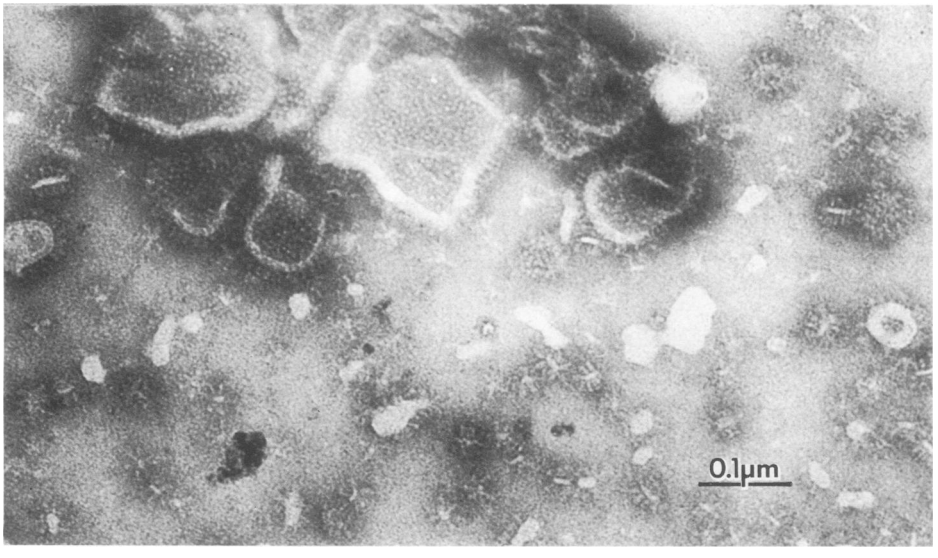

FIG. 5. Electron micrograph of REP of PR8 virus. Negative staining with 2% phosphotungstic acid (pH 7.2).

pothesis that influenza virus hemagglutinin plays an essential role in the interaction between envelopes and cell membranes in virus entry (14, 15, 20). It is interesting to recall that liposomes containing only the hemagglutinin of alphavirus could induce hemolysis (19). However, the present finding is not consistent with evidence of a positive role of influenza virus neuraminidase in membrane fusion (7).

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