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Conformational Changes and Fusion Activity of Influenza Virus Hemagglutinin of the H2 and H3 Subtypes: Effects of Acid Pretreatment

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Marked differences were observed between the H2 and H3 strains of influenza virus in their sensitivity to pretreatment at low pH. Whereas viral fusion and hemolysis mediated by influenza virus X:31 (H3 subtype) were inactivated by pretreatment of the virus at low pH, influenza virus A/Japan/305/57 (H2 subtype) retained those activities even after a 15-min incubation at pH 5.0 and 37°C. Fusion with erythrocytes was measured by using the octadecylrhodamine-dequenching assay with both intact virions and CV-1 monkey kidney cells expressing hemagglutinin (HA) on the plasma membrane. To study the nature of the differences between the two strains, we examined the effects of low-pH treatment on the conformational change of HA by its susceptibility to protease digestion, exposure of the fusion peptide, and electron microscopy of unstained, frozen, hydrated virus. We found that the respective HA molecules from the two strains assumed different conformational states after exposure to low pH. The relationship between the conformation of HA and its fusogenic activity is discussed in the context of these experiments.

Influenza virus invades host cells by attachment to cell surface receptors followed by fusion at low pH in endosomes (14, 17). Fusion is catalyzed by the influenza virus hemagglutinin (HA), a homotrimeric viral spike glycoprotein (29). The HA monomer consists of two disulfide-linked glycopolypeptide chains, HA1 and HA2, which are derived by proteolytic cleavage from the precursor HA0 (13). Below the threshold pH of fusion, HA undergoes a conformational change, resulting in exposure of the hydrophobic amino terminus of HA2 (28), which plays a critical role in fusion (7). However, in the absence of a target membrane, low-pH treatment of various subtypes of influenza virus leads to a conformational change which causes rapid inactivation of viral fusion activity. This phenomenon has been observed with human influenza virus A/PR/8/34 (H1 subtype) (22, 30) and X:47 (H3 subtype) (12, 26), as well as with fowl plague virus, an avian influenza A virus (H7 subtype) (27). In contrast to results with the human H1 and H3 strains, recent studies indicate that pH-dependent fusion induced by HA from influenza virus A/Japan/305/57 (H2 subtype) expressed on the surface of 3T3 fibroblasts with glycophorin-containing liposomes (H. Ellens, J. Bentz, D. Mason, and J. M. White, unpublished observations) or with human erythrocytes (A. Puri and R. Blumenthal, unpublished observations) is not inactivated by pretreatment at low pH.

On the basis of measurements of temperature and pH dependence of the hemolytic activity of influenza virus X:47 and of the rotational mobility of the spike glycoproteins, it was proposed that the mobility of HA is significant for its functional properties (12). In the absence of an apposing membrane, pH-induced conformational changes result in irreversible aggregation of HA in the viral membrane and hence loss of mobility and activity. Since the surface density of HA expressed on the fibroblasts is about 1/10 that found in a virion (Ellens et al., unpublished), this irreversible aggregation leading to inactivation might occur more readily on the intact virus. To examine whether the failure to inactivate the fusion activity of the HA-expressing cells was due to the surface disposition and/or density of the HA or to differences in structure between the human H2 and the H1 or H3 strains, we have studied the effects of low-pH pretreatment on the fusogenic activity of X:31 (H3), a close relative of X:47, as well as A/Japan (H2) HA expressed in both cells and intact virus. We have also examined the effects of low-pH treatment on the conformational change of HA by assessing the exposure of its fusion peptide, measuring its susceptibility to protease digestion, and performing electron microscopy. We conclude that the HA from the two strains assume different conformational states after exposure to low pH.

MATERIALS AND METHODS

Preparation of virus. Influenza viruses X:31 (A/Aichi/68; H3N2) and A/Japan/305/57 (H2N2) were grown in 10-day-old embryonated hen eggs (18). The eggs were infected with 0.1 ml of diluted virus stocks in phosphate-buffered saline (PBS) containing 2 to 5 HA units, incubated at 37°C for 48 h, and then left at 4°C for 12 to 24 h. The virus was harvested and purified by modification of the procedure of Okada (18) (O. Nussbaum, personal communication). The allantoic fluid was centrifuged for 10 min at 1,200 rpm in an RT6000 rotor (Sorvall, Du Pont Instruments, Wilmington, Del.), and then the virus was pelleted by centrifuging the supernatant for 45 min at 90,000 × *g* in an L8-60M ultracentrifuge with an SW28.1 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). The pellets were suspended in a small volume of PBS and dispersed with a Teflon-coated tissue homogenizer (Wheaton Industries, Millville, N.J.). The virus yield from 24 eggs was 6 to 8 mg of protein, determined by using a protein assay reagent kit (Pierce Chemical Co., Rockford, Ill.). Virus preparations (0.5 to 1.0 mg of viral protein per ml)

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were aliquoted and stored at −70°C until further use. The virus was labeled with octadecylrhodamine (R18) as described previously (1, 19), and unincorporated label was removed by centrifugation at 60,000 × *g* for 30 min at 4°C onto a 25% sucrose cushion. The virus was collected and centrifuged in a 1.5-ml polypropylene tube for 3 min at 600 × *g* to remove large aggregates; the final concentration of R18-labeled virus was 0.25 mg of protein per ml.

Interaction of R18-labeled virus with human erythrocytes. Fresh human blood, obtained from the National Institutes of Health blood bank, was washed three times with PBS, diluted to 1% hematocrit, kept at 4°C, and used within 3 days. Virus (150 μl) was added to a 1-ml erythrocyte suspension and gently agitated on a rocker for 30 min at 4°C, after which 9.0 ml of cold PBS was added. The erythrocyte-virus complexes were centrifuged for 5 min at 600 × *g*, washed once with 10 ml of cold PBS, and finally suspended in 0.1 ml of PBS. For hemolysis experiments, virus (1 ml; 0.005 mg/ml in PBS) was incubated with 50 μl of 2% erythrocyte suspension at 4°C for 20 min, after which the erythrocyte-virus complexes were centrifuged at 600 × *g*. Each tube was then incubated for 30 min with 1 ml of PBS adjusted to pH 5.0 and 37°C, after which the erythrocytes were centrifuged and supernatants were assayed for hemoglobin at 520 nm. Maximum hemolysis is defined by measurement of the optical density at 520 nm in the presence of 0.1% Triton X-100.

Interaction of cells expressing surface HA with human erythrocytes. CV1 cells grown to confluence in six-well plates (Costar, Cambridge, Mass.) were washed and overlaid with an appropriate amount of virus in 0.6 ml of Dulbecco modified Eagle medium to ensure that >95% of the cells were infected. Virus was incubated with cells for 1 h at 37°C, after which an additional 2.5 ml of medium was added and the incubation was continued for an additional 4 h. The virus suspension was then replaced by fresh medium, and the cells were incubated for 24 h. For examination of expression of the HA on the surface, the cells were incubated for 15 min with a 0.5% erythrocyte suspension in PBS at room temperature. Unbound cells were removed by washing with PBS, and the percentage of CV1 cells covered by bound erythrocytes was observed under the microscope. Uninfected CV1 cells did not bind any erythrocytes under similar conditions.

Fusion of the HA-expressing cells with R18-labeled erythrocytes was assayed as described previously (16, 21). Briefly, the infected CV1 cells in each well were treated with 2.0 ml of trypsin (5 μg/ml)-neuraminidase (0.5 U/ml) (Sigma Chemical Co., St. Louis, Mo.) in Dulbecco modified Eagle medium for 10 min at room temperature and then washed with the same medium supplemented with 10% fetal calf serum, after which 2 ml of PBS was added. Human erythrocytes (1 ml, 0.05% hematocrit), labeled with R18 as described previously (19, 21), were bound to the cells at room temperature for 5 min. Unbound erythrocytes were removed by washing with 2 ml of PBS. Decorated cells were then lifted from the wells by incubation for 5 min at 37°C in 0.3 ml of trypsin (0.5 mg/ml)-EDTA (0.2 mg/ml) in PBS. Dulbecco modified eagle medium (3 ml) supplemented with 10% fetal calf serum was added to each well, and the cell suspension was transferred to a 5-ml plastic tube. The cells were washed three times by centrifugation with 2 ml of cold PBS. The cell complexes were finally suspended in 0.2 ml of PBS and were placed on ice until further use.

Low-pH pretreatment of virus and cells. A suspension of 0.3 ml of R18-labeled virus was adjusted to a final pH in the range 4.9 to 5.2 and incubated at 37°C for 2 to 15 min, after which the pH was brought back to 7.4 by the addition of Tris. The samples were centrifuged in 1.5-ml polypropylene tubes for 3 min at 600 × *g* to remove large aggregates and was kept at 4°C until further use. HA-expressing cells were pretreated at low pH by overlaying with a low-pH buffer at 37°C for a given period, after which the low-pH buffer was replaced by PBS (pH 7.4).

Spectrofluorometric measurements. Fluorescence dequenching of R18-labeled virus attached to erythrocytes or of R18-labeled erythrocytes attached to HA-expressing cells was measured by using a spectrofluorometer (model 8000; SLM-Aminco, Urbana, Ill.) as described previously (1, 16, 19, 21). The cell suspension was placed in a disposable plastic cuvette at 37°C and pH 7.4 and stirred with a Teflon-coated magnetic stir bar (2 by 8 mm). The pH of the incubation mixture was lowered by the addition of 0.5 M citric acid, and fluorescence was monitored for 400 s, after which Triton X-100 (final concentration, 0.5%) was added to obtain maximum R18 fluorescence. The data were normalized to maximum R18 fluorescence as described previously (1, 16, 19).

Electron microscopy. Virus was diluted to 0.1 mg/ml; incubated for the indicated time, temperature, and pH; and then returned to pH 7.4 by the addition of Tris. It was then concentrated by pelleting for 10 min at 8,000 × *g* and adjusted to about 5 mg/ml. Clumps were dispersed by being drawn repeatedly through a fine Hamilton syringe, and the virus was then applied to a carbon-coated or holey carbon grid, blotted with filter paper, and quench-frozen in liquid ethane in a Reichert KF80 freezing machine. The grid was then transferred to a precooled cooling holder (Gatan Inc., Pleasanton, Calif.) and examined in an electron microscope (model EM 400 T; Philips Electronic Instruments Co., Mahwah, N.J.) under low-dose conditions as described previously (3, 26).

Susceptibility of HA to protease in the intact virus. Untreated or low-pH-treated virus (50 μg in 0.5 ml) was incubated with 30 μg of proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) at 37°C for 30 min at pH 7.4. Before application to the gel, the samples were treated with an equal volume of 20% trichloroacetic acid, and the precipitated protein was sedimented by spinning in an Eppendorf centrifuge for 5 min. The pellets were washed with 1.0 ml of cold ethanol and boiled for 5 min in a buffer containing 0.4% sodium dodecyl sulfate, 5 mM Tris, 20 mM dithiothreitol, and 4% glycerol (pH 6.8). The samples were run on sodium dodecyl sulfate–12.5% polyacrylamide gels under reducing conditions, and the protein bands were stained with Coomassie blue stain.

Exposure of the fusion peptide and proteinase K sensitivity of the BHA fragment. Ectodomain fragments of HA (BHA) from both X:31 and Japan strains were purified, iodinated, and repurified as described previously (28). Immunoprecipitations with anti-fusion peptide antibody were conducted as described previously (28), except that 8 and 15 μl of anti-fusion peptide antiserum was used per 100-μl sample containing ^{125}I-BHA from the X:31 and Japan strains, respectively. The maximum levels of X:31 BHA and Japan BHA precipitated in a single round of immunoprecipitation were 40 and 24%, respectively. Proteinase K digestions were carried out as described previously (4), except that 0.2 mg of proteinase K per ml was used. Following proteinase K digestion, samples were precipitated with 10% trichloroacetic acid. Washed immunoprecipitates and pellets were counted in a gamma counter (Beckman).

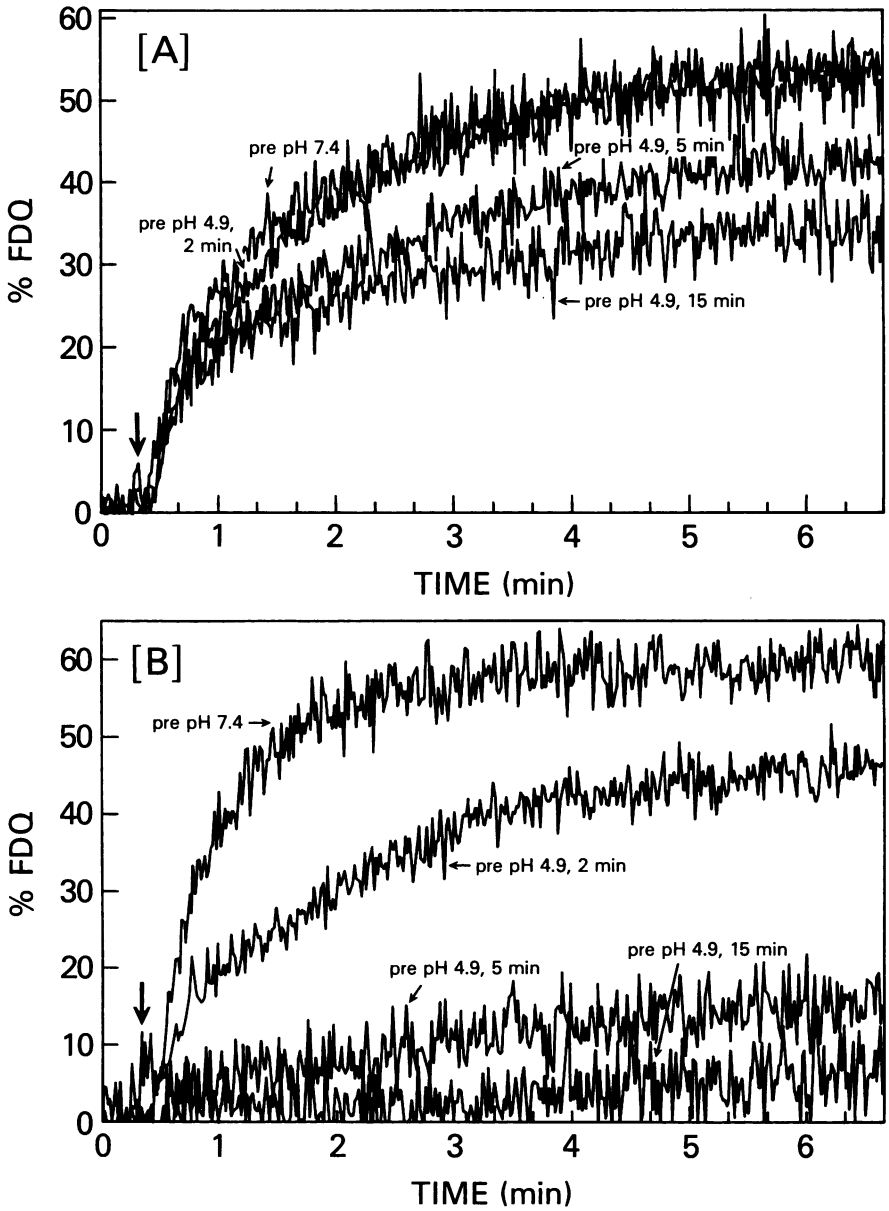

FIG. 1. Kinetics of fluorescence dequenching (FDQ) of R18-labeled erythrocytes with virus-infected cells. CV1 cells were infected with influenza virus A/Japan/305/57 (A) or X:31 (B). The infected cells were pretreated for 15 min at pH 7.4 and 37°C or for different times at pH 4.9 and 37°C, as indicated. R18-labeled erythrocytes were bound to the infected cells, washed, and suspended with trypsin-EDTA as described in Materials and Methods. A 50-μl portion of the erythrocyte-cell complex was injected into 2 ml of PBS (pH 7.4), prewarmed to 37°C. About 20 s later the pH in the medium was changed to 4.9 by addition of 15 μl 0.5 M citrate (arrow). Percent FDQ = $100 \times (F - F_0)/(F_t - F_0)$, where F_0 and F are fluorescence intensities at time zero and at a given time point, respectively, and F_t is the fluorescence after disruption of cells by detergent. The curves for the pH 7.4 controls were completely flat (identical to that shown in Fig. 2A).

RESULTS

HA-induced cell fusion. We used the assay recently developed to directly monitor the kinetics and extent of fusion between virus and cells (1, 10, 19, 26) or between cells (11, 16, 21). The assay is based upon incorporation of the fatty acid probe R18 into intact virus or erythrocytes. The probe is quenched when incorporated at surface densities of >4% into the membrane. Upon fusion with the unlabeled membranes, the probe is diluted, resulting in an increase in fluorescence intensity. HA-induced fusion between R18-labeled erythrocytes and cells expressing HA from the Japan and X:31 strains of influenza virus is shown in Fig. 1A and B, respectively. Upon acidification, the fluorescence increased and leveled off to about 50% fluorescence dequenching in about 3 min at pH 4.9. Pretreatment of cells expressing the Japan HA at low pH did not significantly affect the rates of fusion, although the maximal percent fluorescence dequenching was slightly reduced (Fig. 1A).

In contrast to the results with the Japan strain, incubation of CV1 cells expressing HA from influenza virus X:31 at pH 4.9 for 15 min completely abolished fusogenic activity (Fig. 1B). These results indicate that the different effects of low

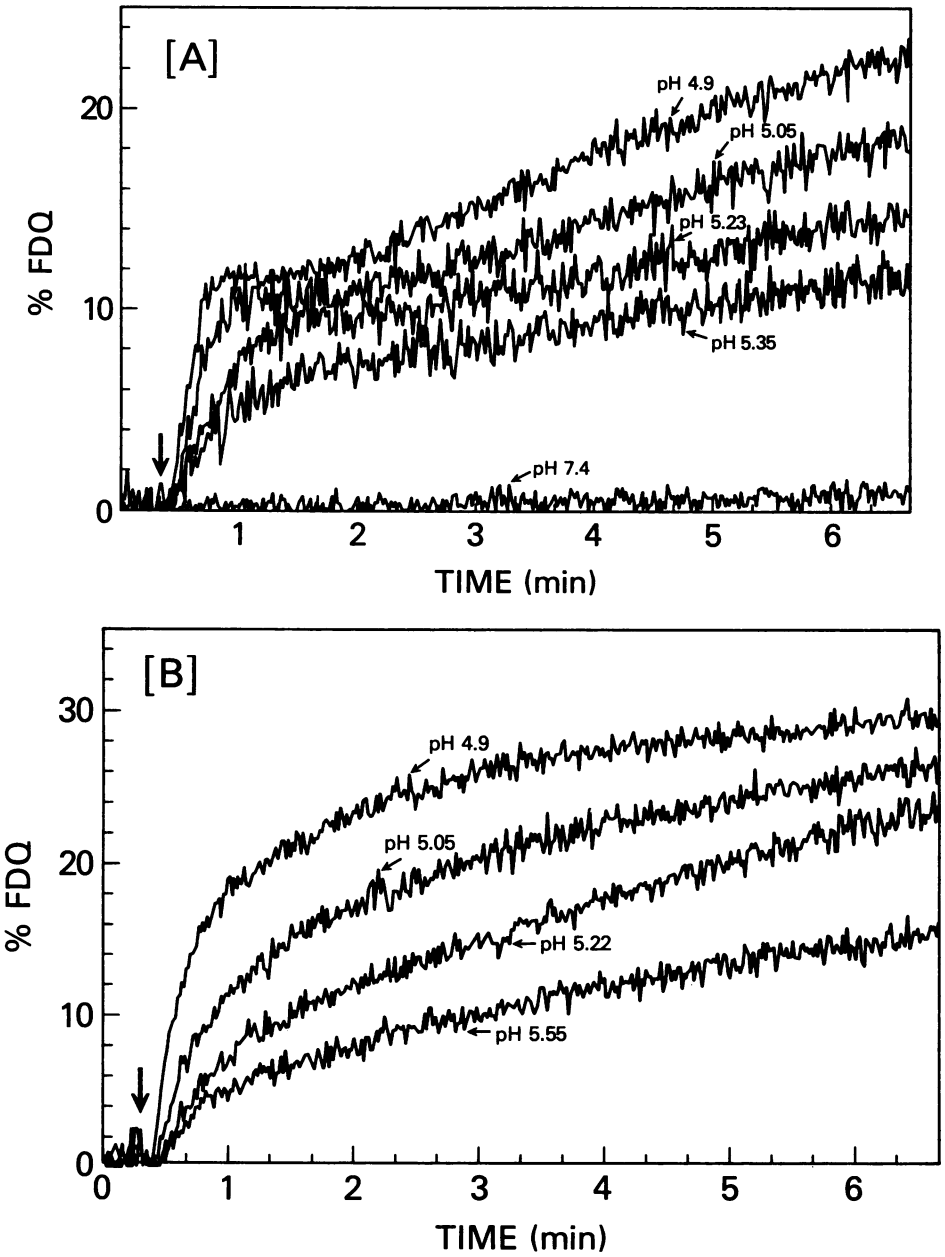

FIG. 2. Kinetics of fluorescence dequenching (FDQ) of R18-labeled virus with erythrocytes. Influenza virus A/Japan/305/57 (A) or X:31 (B) was labeled with R18, bound to erythrocytes, washed, and suspended in PBS. A 50-μl portion of the virus-cell complex was injected into 2 ml of PBS (pH 7.4), prewarmed to 37°C. About 20 s later the pH in the medium was changed by addition of 15 μl of 0.5 M citrate (arrow). The curve for the pH 7.4 control in panel B was completely flat (identical to that shown in Fig. 2A). The percent fluorescence dequenching was calculated as in the legend to Fig. 1.

pH on the fusogenic activity of HA might be due to structural differences between the two strains, rather than to cell surface interactions.

Fusion of intact influenza virus with erythrocytes. To examine whether the failure to inactivate Japan HA by pretreatment at low pH was related to its disposition on the cell surface as compared with intact virus, we studied the fusion of intact virus with erythrocytes by using the R18 dequenching assay. Figure 2 shows fusion of R18-labeled virus from the Japan and X:31 strains with erythrocytes at different pH values. The time course of the increase in fluorescence was biphasic with the intact virion, in that there was a sharp increase upon acidification followed by a slower increase. The slower component appeared after 100 s with the Japan strain (Fig. 2A) and after 20 s with the X:31 strain (Fig. 2B). However, the extent and pH dependence of fluorescence dequenching were about the same for both strains. The biphasic kinetics in Fig. 2 is a special feature of the virus-erythrocyte interaction. It is not seen in the fusion kinetics of HA-expressing cells with erythrocytes or of intact virus with erythrocyte ghosts (data not shown).

To examine the effect of low-pH pretreatment on the fusogenic activity of intact virus, we performed the R18 dequenching assay before and after exposure of both Japan

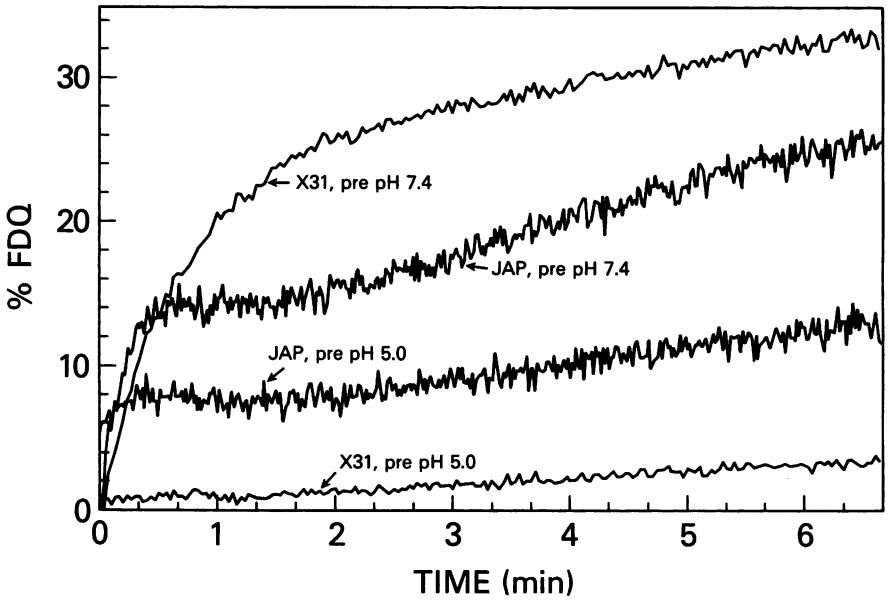

FIG. 3. Effect of acid pretreatment on fusion of influenza virus with erythrocytes. Influenza virus A/Japan/305/57 or X:31 was labeled with R18 and pretreated for 15 min at pH 7.4 and 37°C or at pH 4.9 and 37°C, as indicated. The virus was then bound to erythrocytes, and the fluorescence dequenching assay was performed as described in the legend to Fig. 2. The curves for the pH 7.4 controls were completely flat (identical to that shown in Fig. 2A).

and X:31 virus to low pH (Fig. 3). As expected, fusion of X:31 was completely abolished after 5 min at low pH (12, 26). However, the same treatment did not affect the rate of fusion of the Japan strain, although the maximal percent fluorescence dequenching was reduced by about 50%.

Since the hemolytic activity of influenza virus closely follows its fusogenic activity (22, 26), we compared the hemolytic activities of the two strains after exposure to low pH. The HA titers of low-pH-treated influenza virus from both Japan and X:31 strains decreased 16- to 32-fold (Table 1). Nevertheless, the hemolytic activity of the Japan strain was not affected by low-pH pretreatment even after 30 min at 37°C and pH 4.9. In contrast, the hemolytic activity of the X:31 strains was completely abolished after 2 min at low pH, consistent with earlier findings (12, 26). Therefore, the effects of low-pH pretreatment on the hemolytic activities of the two strains were very similar to the effects on their fusogenic activities as measured by the R18-dequenching assay. We have observed similar inactivation of the hemolytic activity of influenza virus A/PR/8/34 (data not shown), consistent with earlier findings (22, 30).

Conformational changes. To study the nature of the differences between the two strains, we examined the effects of low-pH treatment on the conformational change of HA by different techniques. Whereas the neutral conformation of HA is resistant to a variety of proteases, the acid forms are susceptible to digestion with trypsin, proteinase K, and other proteases (4, 25). The pH dependence for conversion to protease sensitivity is similar to that for the fusogenic activity of HA (7). We have used this technique to study the conformational change of HA from the Japan and X:31 strains. Figure 4 shows sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels of intact virus before and after treatment with proteinase K. HA1 was clearly visible in the untreated virus after exposure to the enzyme. However, low-pH treatment of the virus followed by proteinase K digestion led to the disappearance of HA1 from both virus strains.

TABLE 1. Hemagglutination and hemolysis of human erythrocytes by influenza virus

Virus	pH used[a]	HAU	% Hemolysis[b]
Japan	7.4	256	37.5
Japan	4.9[c]	8	49.1
X:31	7.4	512	52.3
X:31	4.9[c]	32	1.9

[a] Virus was incubated for 15 min and 37°C at the indicated pH.
[b] Mean values of two determinations. Variation between the two values was less than 5%. Similar results were found in two independent experiments with purified virus and four experiments conducted with allantoic fluids.
[c] The pH was returned to 7.4 after acid pretreatment.

Treatment of intact influenza virus with the enzyme bromelain results in the release of nearly the entire N-terminal ectodomain of HA (termed BHA) in a water-soluble form (25). Iodinated BHA from both Japan and X:31 strains rapidly became sensitive to proteinase K with similar kinetics after exposure to low pH and 37°C (Fig. 5A) as had been reported previously (5, 7).

Conformational transitions of HA have also been probed by using anti-HA peptide antibodies which recognize known regions of the protein (28). Using an antibody against HA2 residues 1 through 29 (which include the fusion peptide) from X:31 HA, White and Wilson (28) have shown that the fusion peptide comes out of the trimer interface at pH < 6. Since the sequences of the Japan and X:31 HA2 residues 1 through 29 are quite homologous (6), we could use the same anti-peptide antibody to examine the low-pH-induced release of the fusion peptide from the BHA of both strains. The fusion peptide of both the X:31 and Japan strains became exposed within 1 min after the pH had been lowered (Fig. 5B). These results strongly suggest an irreversibly altered conformation of Japan HA after pretreatment at low pH.

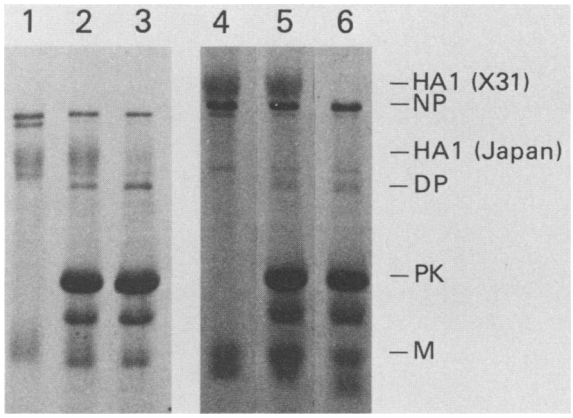

FIG. 4. Effect of acid pretreatment on the susceptibility of influenza virus to proteolytic digestion. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels stained with Coomassie blue of influenza virus A/Japan/305/57 (lanes 1 through 3) or X:31 lanes (4 through 6). Lanes: 1 and 4, untreated virus; 2 and 5, virus preincubated at pH 7.4 and treated with proteinase K; 3 and 6, virus preincubated at pH 4.9 and 37°C and treated with proteinase K. Abbreviations: NP, nucleoprotein; M, matrix protein; DP, digestion products; PK, proteinase K.

Electron microscopy. Electron microscopy of negatively stained as well as unstained, frozen, hydrated influenza virus has revealed that the HA spikes in the intact virion at neutral pH are regular rectangular projections about 13 nm in length (20). Acid treatment of the X:31 strain results in a disordered appearance (20, 26). We examined the HA spikes on virions of both strains in the unstained, frozen, hydrated state after exposure to low pH for 15 min at 37°C. In accordance with previous observations, the spikes of the X:31 strain treated at low pH for 15 min at 37°C became completely disorganized (Fig. 6D). Other structural reorganizations occurred, which became more pronounced after longer incubations at low pH (data not shown). On the other hand, after a 15-min treatment at low pH and 37°C, virus particles of the Japan strain did not show significant morphological changes (Fig. 6B). We observed exactly the same pattern as in Fig. 6B after a 15-min treatment at low pH and 37°C without reversal to pH 7.4. However, after several days the morphology of the HA spikes of the acid-treated Japan strain became disordered and similar to those of the X:31 strain.

DISCUSSION

A variety of viruses enter cells by acid-activated fusion following endocytosis (14, 17, 27a). Low-pH pretreatment of some viruses in this group (e.g., orthomyxovirus [12, 22, 26, 30], flavivirus [8], and alphavirus [5, 15]) renders them noninfectious by abolishing their fusion activity. On the other hand, low-pH pretreatment of vesicular stomatitis virus, a rhabdovirus, enhances its ability to penetrate cells by membrane fusion (19). In this study we found that low-pH-induced inactivation is not a general phenomenon for all orthomyxoviruses. It appears that at least one influenza virus of the H2 subtype is not inactivated by low-pH pretreatment. It has been shown that after treatment of different strains of influenza A virus at low pH, the threshold pH (at which infectivity is lost) is dependent on the HA subtype of the virus strain (24). H1, H5, and H7 strains are relatively labile (pH threshold 5.6 to 6.0), H3 is slightly more stable (pH threshold 5.1 to 5.4), whereas the infectivity of

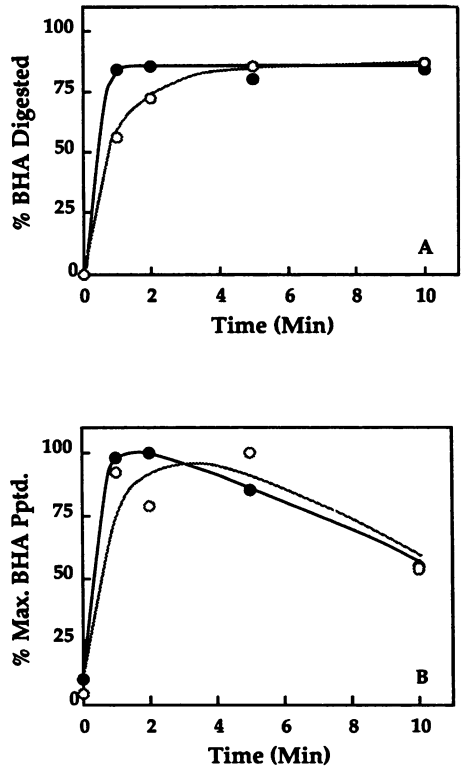

FIG. 5. Kinetics of conformational changes of isolated HA from influenza virus. Samples (100 μl) containing 10,000 cpm of ^{125}I-BHA from X:31 (●) or Japan (○) strains of influenza virus were acidified to pH 5 at 37°C for the indicated times, neutralized, and then digested with proteinase K (A) or immunoprecipitated with anti-fusion peptide antiserum (B) as described in Materials and Methods.

H2 was destroyed only at pH <4.6 (24), consistent with the data in the present study. These results may provide an explanation for the observation (9) that H2 virus strains replicate to high titers in birds after oral inoculation whereas H3 strains do not. Presumably the H2 strains are not inactivated by the low pH adjacent to cells lining the intestinal tract. We found that acid pretreatment of the Japan strain (H2) of influenza virus did not affect its infectivity in embryonated hen eggs (data not shown).

Using a direct assay for membrane fusion, we observed similar effects of the acid pretreatment for HA expressed in cells or in the intact virus. Whereas the rate of fusion mediated by HA decreased rapidly upon acid treatment in X:31, low-pH pretreatment had no effect on the rate of fusion mediated by the Japan HA. The extent of Japan HA-mediated fusion monitored by the R18-dequenching assay was, however, partially acid inactivated. Since there were virtually no morphological changes in the overall population of acid-treated Japan virus (Fig. 6), it is unlikely that the decrease in extent is due to a heterogeneous population of virus. Possible causes for the changes in extents are virus aggregation or binding to nonfusogenic sites. These factors may be more pronounced with intact virus than with HA-expressing cells (Fig. 1A and 3).

We found that binding of the Japan strain to cells was unchanged after acid pretreatment, which is consistent with the observation (23) that the K_d of HA did not change after acid treatment. However, in contrast, the hemagglutination titers of the Japan strain decreased after acid treatment

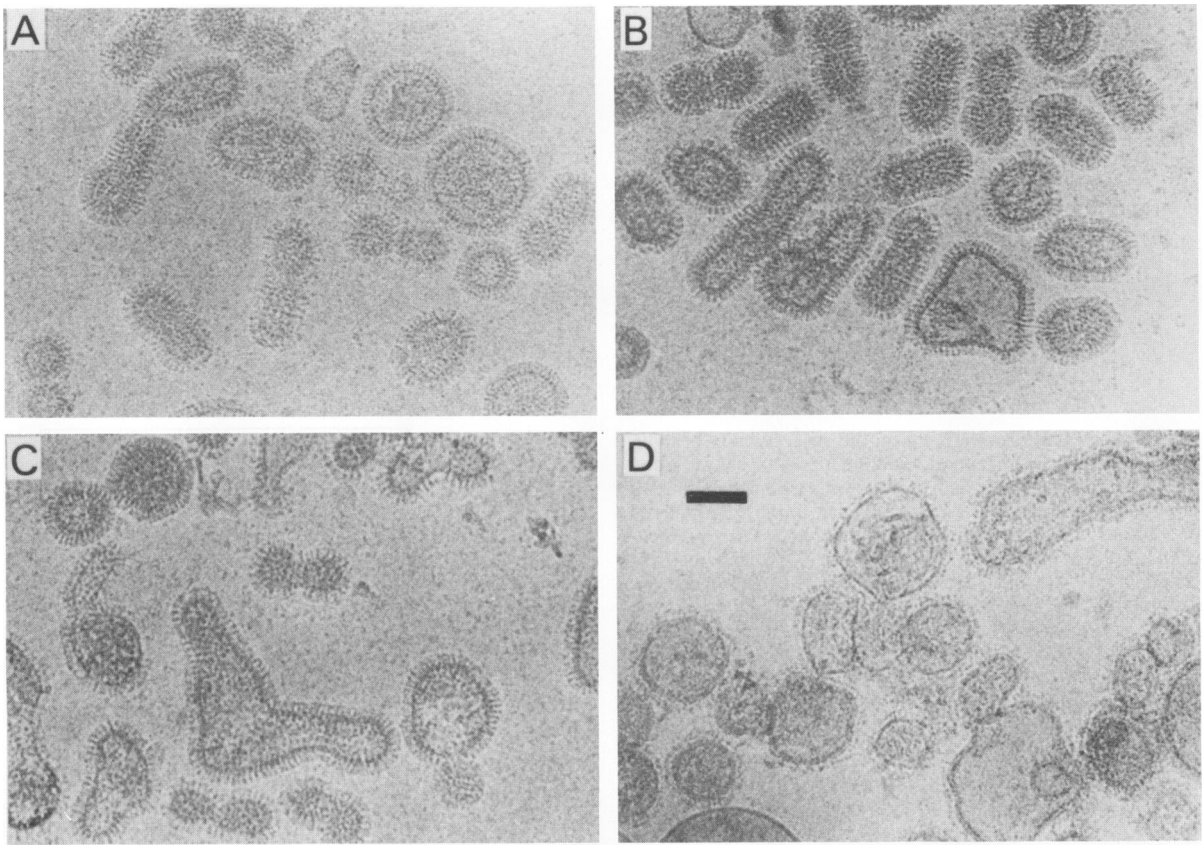

FIG. 6. Electron micrographs (100 kV) of unstained, frozen, hydrated influenza virus. (A and B) Virus from the Japan strain; (C and D) virus from the X:31 strain. Panels A and C contain virus at pH 7.4 and 37°C; panels B and D contain virus incubated for 15 min at pH 4.9 and 37°C and subsequently neutralized.

(Table 1). It has been shown that the inactivation of influenza virus from the X:47 strain (a close relative of the X:31 strain) is a result of aggregation of HA envelope proteins on the viral surface (12). In the absence of an apposing membrane, aggregation of HA in the viral membrane does not occur in the Japan strain (Fig. 6). A possible explanation for the decrease in hemagglutination titers of acid-treated Japan strain could be that binding of HA to the erythrocyte surface sialoglycoproteins causes aggregation of HA in the viral membrane. In the case of acid-treated HA, the self-aggregation of HA could be more pronounced than in the untreated virus. Self-aggregation would leave little HA available for cross-linking with other erythrocytes and would therefore lead to a decrease in hemagglutinating activity. This interpretation could also be an explanation for the decrease in extent of fusion with the acid-treated Japan strain.

TABLE 2. States of influenza virus HA

Determination method	Definition of state			Reference
	Neutral (T)	Intermediate (R)	Low pH (A)	
Anti-peptide antibody reactivity to				
Loop	−	+	+	28
Fusion	−	+	+	28
COOH terminus of HA1	−	+	+	28
Interface	−	−	+	28
Hinge	−	−	+	28
Electron microscopy	Spike	Spike	Fuzzy	20, 26
pH inactivation	−	−	+	12, 22, 26, 30
Protease sensitivity	−	+	+	4, 7, 26
Liposome binding	−	+	+	4, 7, 26
Rotational mobility	+	ND[a]	−	12
Hemagglutination	+	−	−	This study
Hemolysis	−	ND	ND	This study
Fusion	−	ND	ND	This study

[a] ND, Not determined.

It appears from this study that inactivation is unrelated to the cell surface disposition of HA. The HA of the H2 strain in intact virus, or expressed in cells, was not inactivated by low-pH pretreatment, whereas that of the H3 strain was. Instead, it appears that the difference is related to the nature of pH-induced conformational changes. Although the sequence of the fusion peptide and the location of cysteine residues are conserved, there is only about 45% overall sequence homology between the two strains (6).

There seems to be an apparent contradiction between the high rate of conformational change of Japan HA, as measured by the proteinase K and antipeptide antibody assays (Fig. 5), and the lack of inactivation. This can be reconciled by considering different states of HA induced by low pH. Extensive studies have been performed on the acid-triggered conformational change that is related to the fusogenic activity of influenza virus HA. Models have been proposed in which the heads of HA come apart but remain globular, the stem region remains trimeric, and there is no major change in overall secondary structure (29). White and Wilson (28) have probed the details of the pH-dependent conformational changes in the X:31 HA by using a panel of anti-HA-peptide antibodies. The results of the study indicate that the acid-triggered conformational change of isolated HA occurs in at least two steps: the fusion peptide comes out of the trimer interface (intermediate state), and then the globular heads dissociate.

In their model for acid-triggered conformational change, White and Wilson (28) introduced an "intermediate state" in which the fusion peptide as well as residues near the COOH terminus of HA1 are exposed. However, release of the fusion peptide and exposure of the COOH terminus of HA1 exhibit different kinetics and pH dependence (28), indicating that more than one intermediate state may exist. It appears that X:31 HA becomes proteinase K sensitive coincident with exposure of the COOH terminus of HA1 (J. M. White, unpublished observations). For simplicity, we identify the same intermediate state by the different assays (i.e., release of fusion peptide, exposure of the COOH terminus of HA1, proteinase K sensitivity, and liposome binding) (see Table 2). The data in the present study indicate that the Japan HA undergoes the transition to the intermediate state only at low pH and 37°C, as shown by the proteinase K sensitivity and antibody-binding assays (Fig. 5), although the heads are still together according to the unaltered spike morphology shown in Fig. 6B. By contrast, the X:31 HA undergoes both steps at low pH and 37°C, as indicated by the biochemical assays (Fig. 5) and the disordered spike morphology (Fig. 6D). On the other hand, at 0°C and low pH the X:47 HA (a close relative of the X:31 HA) will undergo only the first step, as indicated by protease sensitivity and liposome-binding assays (26). However, at 0°C, low-pH-treated X:47 HA maintains its spike morphology (26). It also appears that the fusion peptide of HA is rapidly released from X:31 HA at 4°C and pH 5 (T. Stegmann, J. M. White, and A. Helenius, submitted for publication).

A three-state model has been proposed which relates the conformational transitions of HA to mechanisms of viral fusion (2). The proposed model for pH-induced activation of HA is analogous to ligand-induced activation of allosteric enzymes or receptor-linked ion channels. According to the model, HA undergoes a proton-driven shift from the T (tense) state at neutral pH, to the R (relaxed) state, followed by an opening up to the A (active) state. Those three states may be equated to the three states identified in the antipeptide antibody studies of White and Wilson (28). Table 2 shows a summary of the way the different states have been defined.

How does this relate to viral fusion and inactivation? One possible hypothesis is that fusion will occur during a transition from the R to the A state, while the HA is associated with the target membrane. Such a transition in the absence of the target membrane will presumably lead to inactivation, as is the case with the X:31 HA. On the other hand, in the absence of target membranes, the Japan HA will undergo the transition to the R state only at 37°C and low pH, and will therefore remain active. We surmise that the Japan HA will undergo the fusogenic transition to the A state only when bound to the target. Further experiments are needed to test this hypothesis.

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LITERATURE CITED

1. **Blumenthal, R., A. Bali-Puri, A. Walter, D. Covell, and O. Eidelman.** 1987. pH-dependent fusion of vesicular stomatitis virus with Vero cells: measurement by dequenching of octadecylrhodamine fluorescence. J. Biol. Chem. **262**:13614–13619.
2. **Blumenthal, R., A. Puri, D. P. Sarkar, Y. Chen, O. Eidelman, and S. J. Morris.** 1988. Membrane fusion mediated by viral spike glycoproteins. UCLA Symp. Mol. Cell. Biol. **90**:197–217.
3. **Booy, F. P., R. W. Ruigrok, and E. F. van Bruggen.** 1985. Electron microscopy of influenza virus. A comparison of negatively stained and ice-embedded particles. J. Mol. Biol. **184**: 667–676.
4. **Doms, R. W., A. Helenius, and J. White.** 1985. Membrane fusion activity of the influenza virus hemagglutinin. The low pH-induced conformational change. J. Biol. Chem. **260**:2973–2981.
5. **Edwards, J., E. Mann, and D. T. Brown.** 1983. Conformational changes in Sindbis virus envelope proteins accompanying exposure to low pH. J. Virol. **45**:1090–1097.
6. **Gething, M. J., J. Bye, J. Skehel, and M. Waterfield.** 1980. Cloning and DNA sequence of double-stranded copies of haemagglutinin genes from H2 and H3 strains elucidates antigenic shift and drift in human influenza virus. Nature (London) **287**:301–306.
7. **Gething, M. J., R. Doms, D. York, and J. White.** 1986. Studies on the mechanism of membrane fusion: site-specific mutagenesis of the hemagglutinin of influenza virus. J. Cell Biol. **102**: 11–23.
8. **Gollins, S. W., and J. S. Porterfield.** 1986. The uncoating and infectivity of the flavivirus West Nile on interaction with cells: effects of pH and ammonium chloride. J. Gen. Virol. **67**: 1941–1950.
9. **Hinshaw, V. S., R. G. Webster, C. W. Naeve, and B. R. Murphy.** 1983. Altered tissue tropism of human-avian reassortant influenza viruses. Virology **128**:260–263.
10. **Hoekstra, D., T. de Boer, K. Klappe, and J. Wilschut.** 1984. Fluorescence method for measuring the kinetics of fusion between biological membranes. Biochemistry **23**:5675–5681.
11. **Hoekstra, D., and K. Klappe.** 1986. Use of a fluorescence assay to monitor the kinetics of fusion between erythrocyte ghosts, as induced by Sendai virus. Biosci. Rep. **6**:953–960.
12. **Junankar, P. R., and R. J. Cherry.** 1986. Temperature and pH dependence of the haemolytic activity of influenza virus and of the rotational mobility of the spike glycoproteins. Biochim. Biophys. Acta **854**:198–206.
13. **Klenk, H. D., R. Rott, M. Orlich, and J. Blodorn.** 1975. Activation of influenza A viruses by trypsin treatment. Virology **68**:426–439.
14. **Marsh, M., and A. Helenius.** 1989. Virus entry into animal cells. Adv. Virus Res. **36**:107–151.

15. **McClure, M. O., M. Marsh, and R. A. Weiss.** 1988. Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism. EMBO J. **7**:513–518.
16. **Morris, S. J., D. P. Sarkar, J. M. White, and R. Blumenthal.** 1989. Kinetics of pH-dependent fusion between 3T3 fibroblasts expressing influenza hemagglutinin and red blood cells: measurement by dequenching of fluorescence. J. Biol. Chem. **264**: 3972–3978.
17. **Ohnishi, S.** 1988. Fusion of viral envelopes with cellular membranes. Curr. Top. Membr. Transp. **32**:257–296.
18. **Okada, Y.** 1962. Analysis of giant polynuclear cell formation caused by HVJ virus from Ehrlich ascites tumor cells. Exp. Cell Res. **26**:98–107.
19. **Puri, A., J. Winick, R. J. Lowy, D. Covell, O. Eidelman, A. Walter, and R. Blumenthal.** 1988. Activation of vesicular stomatitis virus fusion with cells by pretreatment at low pH. J. Biol. Chem. **263**:4749–4753.
20. **Ruigrok, R. W., N. G. Wrigley, L. J. Calder, S. Cusack, S. A. Wharton, E. B. Brown, and J. J. Skehel.** 1986. Electron microscopy of the low pH structure of influenza virus haemagglutinin. EMBO J. **5**:41–49.
21. **Sarkar, D. P., S. J. Morris, O. Eidelman, J. Zimmerberg, and R. Blumenthal.** 1989. Initial stages of influenza hemagglutinin-induced cell fusion monitored simultaneously by two fluorescent events: cytoplasmic continuity and lipid mixing. J. Cell Biol. **109**:113–122.
22. **Sato, S. B., K. Kawasaki, and S. Ohnishi.** 1983. Hemolytic activity of influenza virus hemagglutinin glycoproteins activated in mildly acidic environments. Proc. Natl. Acad. Sci. USA **80**:3153–3157.
23. **Sauter, N. K., M. D. Bednarski, B. A. Wurzburg, J. E. Hanson, G. M. Whitesides, J. J. Skehel, and D. C. Wiley.** 1989. Hemagglutinins from two influenza virus variants bind to sialic acid derivatives with millimolar dissociation constants: a 500-MHz proton nuclear magnetic resonance study. Biochemistry **28**: 8388–8396.
24. **Scholtissek, C.** 1985. Stability of infectious influenza A viruses at low pH and at elevated temperature. Vaccine **3(Suppl. 3)**: 215–218.
25. **Skehel, J., P. Bayley, E. Brown, S. Martin, M. Waterfield, J. White, I. Wilson, and D. Wiley.** 1982. Changes in conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. Proc. Natl. Acad. Sci. USA **79**: 968–972.
26. **Stegmann, T., F. P. Booy, and J. Wilschut.** 1987. Effects of low pH on influenza virus. Activation and inactivation of the membrane fusion capacity of the hemagglutinin. J. Biol. Chem. **262**:17744–17749.
27. **White, J., J. Kartenbeck, and A. Helenius.** 1982. Membrane fusion activity of influenza virus. EMBO J. **1**:217–222.
27a. **White, J. M.** 1990. Viral and cellular membrane fusion proteins. Annu. Rev. Physiol. **52**:675–697.
28. **White, J. M., and I. A. Wilson.** 1987. Anti-peptide antibodies detect steps in a protein conformational change: low-pH activation of the influenza virus hemagglutinin. J. Cell Biol. **105**: 2887–2896.
29. **Wiley, D. C., and J. J. Skehel.** 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. **56**:365–394.
30. **Yewdell, J. W., W. Gerhard, and T. Bachi.** 1983. Monoclonal anti-hemagglutinin antibodies detect irreversible antigenic alterations that coincide with the acid activation of influenza virus A/PR/834-mediated hemolysis. J. Virol. **48**:239–248.