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Monoclonal Anti-Hemagglutinin Antibodies Detect Irreversible Antigenic Alterations That Coincide with the Acid Activation of Influenza Virus A/PR/834-Mediated Hemolysis

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Exposure of influenza virus to an acidic environment, which is known to be required for viral fusion and hemolysis, has recently been shown to induce a conformational change in the hemagglutinin molecule. In the present study, we examined the effects of acid incubation on the antigenicity, biological activity, and morphology of influenza virus A/PR/8/34 (H1N1). Incubation of PR8 virus at pH 5 in the absence of erythrocytes resulted in a rapid and irreversible loss of viral hemolytic activity and infectivity. Apart from a less distinct appearance of the viral surface projections and slight damage to the envelope structure, acid incubation did not result in gross morphological changes in the viral architecture. The acid-induced change could be detected in the form of greatly increased or decreased binding of many monoclonal antibodies directed to each of the four major antigenic regions of the hemagglutinin. Triggering of viral hemolytic activity and antigenic alterations was similarly pH dependent. In addition, the different pH dependencies of egg-grown and trypsin-treated MDCK-grown viruses coincided with an analogous pH dependence of the antigenic alterations that were observed with these viruses. These observations are compatible with the idea that some of the anti-hemagglutinin antibodies detect conformational changes in the hemagglutinin which are required for the initiation of fusion and hemolysis.

Influenza viruses, like other enveloped viruses, can fuse with cellular membranes and mediate hemolysis and cell fusion. These activities are only observed, however, when the ambient pH is sufficiently acid (generally between pH 5 and 6) (8–10, 16). This phenomenon appears to be biologically relevant since current evidence suggests that influenza virus enters host cells after endocytosis and transfer to lysosomes, at which point the low pH triggers uncoating by fusion with the lysosomal membrane (11–13, 18).

It is now well established that the viral hemagglutinin (HA) mediates the fusion process (20). In addition, it has been shown repeatedly that proteolytic cleavage of the HA into two disulfide-linked subunits (termed HA1 and HA2) by trypsin or cellular trypsin-like proteases (9, 10) is necessary for the expression of maximal fusion activity (8, 11, 20). This cleavage generates a hydrophobic N-terminal region on the HA2 which shows high sequence homology with an analogous polypeptide region on the paramyxovirus fusion protein (7) and which is thought to be involved in the fusion process.

Recently, Skehel and collaborators reported that bromelain-released HA, which lacks the carboxy-terminal HA2 residues that anchor the HA into the viral membrane, undergoes irreversible conformational alterations when treated with pH 5 buffer, as manifested by autoaggregation, increased binding to lipid vesicles and detergent, increased susceptibility to tryptic cleavage, and changes in the circular dichroism spectra (18). The present study shows that marked irreversible changes in HA antigenicity, detectable by anti-HA hybridoma antibodies, accompany acid activation of hemolytic activity and that these changes are not due to acid-induced aggregation or disintegration of virions.

MATERIALS AND METHODS

Virus and purified HA. Influenza virus PR8 [A/PR/8/34 (H1N1)] was grown in the allantoic cavity of embryonated hen eggs and purified as previously described (4). Virus titers were determined by hemagglutination assay as described by Fazekas de St. Groth and Webster (3) and are expressed as HA units (HAU) per milliliter. Bromelain-solubilized HA (BHA) was produced by partial digestion of purified PR8 virus with bromelain as described by Brand and Skehel (1), followed by removal of cosolubilized neuraminidase by passage over a column containing immobilized monoclonal anti-neuraminidase antibody.

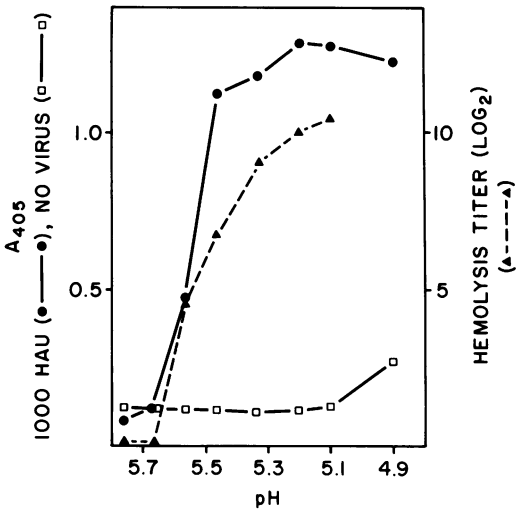

FIG. 1. pH dependence of PR8 virus-induced hemolysis. Human erythrocytes were incubated, with or without virus, for 1.5 h at 37°C in 0.15 M sodium citrate buffer at the indicated pH, and the amount of hemoglobin released into the medium was determined by measurement of absorptivity at a 405-nm wavelength. Symbols: □, A_{405} in the absence of PR8 virus; ●, A_{405} in the presence of 1,000 HAU of PR8 virus; ▲, hemolysis activity of PR8 virus at the indicated pH values, determined as described in the text.

Antibodies. Hybridoma anti-HA antibodies were produced and characterized as previously described (2, 5, 6).

HI titrations. HA inhibition (HI) titrations were performed in 96-well polystyrene microtiter plates (Linbro, Flow Laboratories, Inc., Hamden, Conn.). Serial twofold dilutions of hybridoma antibody-containing ascites fluids in 25 μl of HAS (phosphate-buffered saline [pH 7.2], 0.04% NaN_3) were mixed with four agglutinating doses of virus in 25 μl of HAS and incubated for 60 min at room temperature. Human erythrocytes (1% [vol/vol] in HAS, 50 μl per well) were then added to the virus-antibody mixture and incubated for 45 min at room temperature. The HI titer is expressed as the reciprocal of the highest antibody dilution inhibiting three agglutinating doses of virus.

RIA. Purified virus (20 HAU) in 50 μl of HAS or 250 ng of BHA in 50 μl of 0.02 M NaCl was adsorbed overnight to wells of round-bottom polyvinyl plates (Costar, Cambridge, Mass.), washed twice with HAS, and incubated for 2 h at room temperature with 100 μl of 0.15 M sodium citrate–acetate buffer (pH 7) or 0.15 M sodium citrate buffer (pH 5). After being washed twice with HAS, plates were incubated with 50 μl of 1% bovine serum albumin–HAS for 2 h and then used for radioimmunoassay (RIA) as described previously (5).

Hemolysis titrations. Hemolysis titrations were performed in 96-well round-bottom polystyrene microtiter plates. Serial twofold virus dilutions in 25 μl of 0.15 M sodium chloride were mixed with 25 μl of human erythrocytes (2% [vol/vol] in 0.15 M sodium chloride) and 100 μl of 0.15 M sodium citrate buffer at various pHs and incubated for 1.5 h at 37°C. The plates were then centrifuged at 800 × *g* for 5 min, and 100 μl of supernatant was transferred to flat-bottom 96-well polystyrene plates. The amount of hemoglobin in the supernatants was determined from the absorbance at 405 nm (A_{405}), as measured by an optical plate reader (Titertek Multiscan, Flow Laboratories, Inc., McLean, Va.). The hemolysis titer is expressed as the reciprocal virus dilution yielding 25% hemolysis as given by the formula: % hemolysis = $\{[A_{405}$ (experimental) − A_{405} (no virus added)]/[A_{405} (maximal) − A_{405} (no virus added)]$\}$ × 100.

Electron microscopy. Virus diluted with HAS to a concentration of 1,000 HAU/ml was applied to carbon-coated grids. The virus was treated by allowing the virus-containing grids to float for 15 min at 37°C on a drop of 0.15 M sodium citrate (pH 5.0) or HAS (pH 7.2). The incubation was stopped by transferring the grids to a drop of HAS for neutralization. This was immediately followed by immune reactions for 30 min at room temperature, which were done by floating the grids on a drop of antibody-containing ascites fluid diluted with HAS. After the grids were washed for 5 min with HAS, the material was negatively stained with a 2% solution of phosphotungstic acid. The specimens were examined in a Philips 400 electron microscope.

RESULTS

pH dependence of the hemolytic activity of PR8 virus. The pH dependence of PR8 [A/PR/8/34 (H1N1)] virus-mediated hemolysis was examined by measuring the amount of hemoglobin released from human erythrocytes after 1.5 h of incubation at 37°C in media of various pHs in the absence and presence of PR8. In the absence of virus, some spontaneous release of hemoglobin occurred in the low-pH range (Fig. 1). This spontaneous release remained low provided that fresh human erythrocytes were used. In the presence of 1,000 HAU of virus, hemolysis became detectable at pH 5.6 and reached near the maximum level at pH 5.4 (Fig. 1). This is in good agreement with previous reports of the pH dependence of PR8 virus-mediated hemolysis (8, 13). To quantitate the viral hemolytic activity, the virus dose inducing 25% of the maximal hemolysis level was determined at each pH value. The resulting hemolysis activity curve reached maximum values around pH 5.1 to 5.0. In this pH range, roughly 1 HAU of PR8 induced 25% hemolysis in a 1.5-h incubation period.

Effect of pretreatment of virus at pH 5 on viral biological activities and antigenicity. Recently, Skehel and collaborators (18) reported that treatment of purified influenza virus HA at pH 5 leads to irreversible conformational alterations detectable by changes in circular dichroism spectra, differences in susceptibility to tryptic cleavage, and in certain physical properties. In an attempt to search for additional manifestations of the acid-induced alterations, PR8 virus was first incubated in the absence of erythro-

cytes at pH 5 and subsequently was either directly added to erythrocytes at pH 5 for determination of the viral hemolytic activity or was readjusted to pH 7 for determination of the viral hemagglutination titer and reactivity in the HI test with a panel of monoclonal anti-HA antibodies.

The hemolytic activity of PR8 decayed rapidly during pretreatment (at 20 to 24°C) of the virus at pH 5; more than 95% of the original activity was lost after incubation for 1.5 h (Fig. 2). By contrast, the hemagglutination activity of the virus showed no decay. Thus, the observed loss in hemolytic activity was not due to an inability of the pH 5-treated virus to adsorb to erythrocytes. Rather, these findings indicated that the acid-induced alterations had specifically affected the ability of the virus to mediate hemolysis.

PR8 virus was next examined in the HI test after pH 5 treatment (2 h at room temperature) for its reaction with a panel of 44 monoclonal anti-HA hybridoma antibodies. The monoclonal antibodies are grouped (Fig. 3) according to the antigenic region ("site") on the HA to which they bind. The site specificity was determined previously on the basis of the reaction of these antibodies with 44 structurally defined laboratory PR8 HA virus mutants (2, 6). For reference purposes, Fig. 3 also gives the designations used by Wiley et al. for the corresponding sites on the H3 subtype of HA (22). Three major points are evident from these HI tests. First, the majority

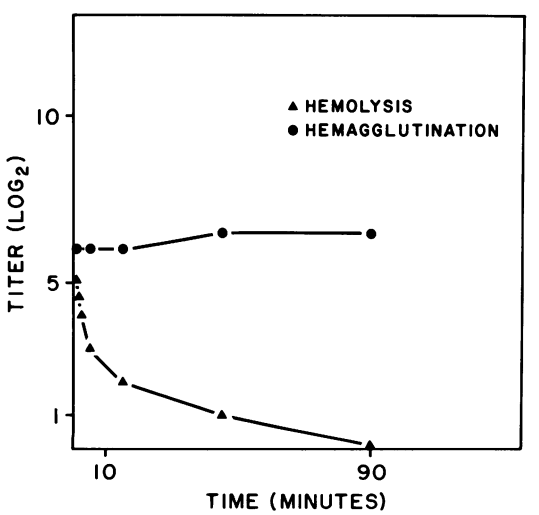

FIG. 2. Kinetics of the decay of hemolysis activity of PR8 virus during incubation in pH 5 buffer. Virus was incubated at room temperature for various periods of time in 0.15 M sodium citrate buffer at pH 5 and then either was added directly at various dilutions to human erythrocytes in pH 5 buffer for a determination of the hemolysis titer (see the text) or was adjusted to pH 7 and assayed for the hemagglutination titer.

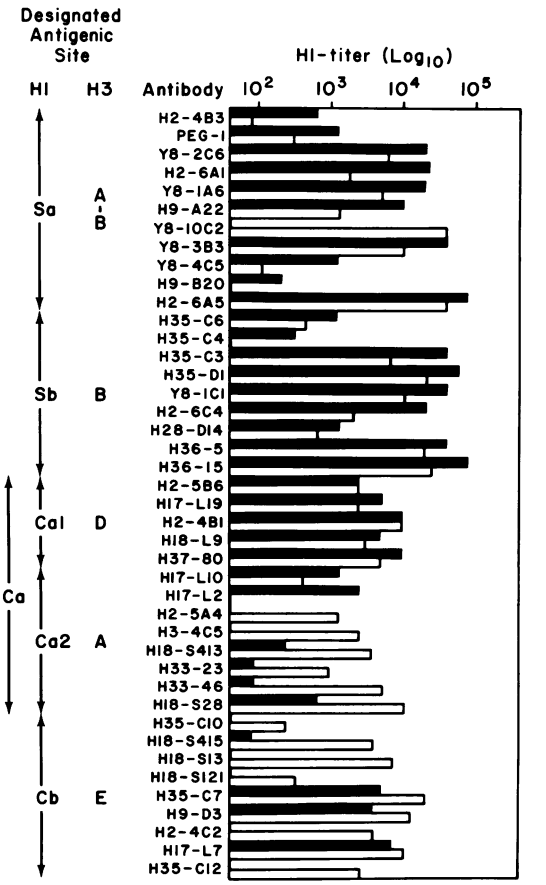

FIG. 3. Comparison of the HI activity of antibodies against PR8 virus before and after treatment with pH 5 buffer. PR8 virus was incubated for 2 h at room temperature in sodium citrate buffer (pH 5) and was then readjusted to pH 7. Monoclonal antibodies (ascitic fluids) were then tested in parallel for HI titer against four agglutinating doses of PR8 virus before (■) and after (□) treatment with pH 5 buffer. HI titers are expressed as the reciprocal of the antibody dilution ($\log_{10}$) at which approximately three agglutinating doses of virus were inhibited from agglutinating human erythrocytes.

(70%) of the antibodies exhibited significant changes (≥-fourfold) in HI titers against pH 5-treated virus compared with untreated virus. These changes cannot be explained by alterations in the avidity of pH 5-treated virus for human erythrocytes since both increases and decreases in the HI titer were observed. Second, significant changes in the HI titer occurred within each antibody specificity group. Thus, acid treatment apparently led to widespread alterations in the HA. Third, the type of HI change was related, in general, to the site of the HA to which the antibodies bind. For instance, antibodies to sites Sa and Sb, with one exception, had reduced HI activity against pH 5-treated

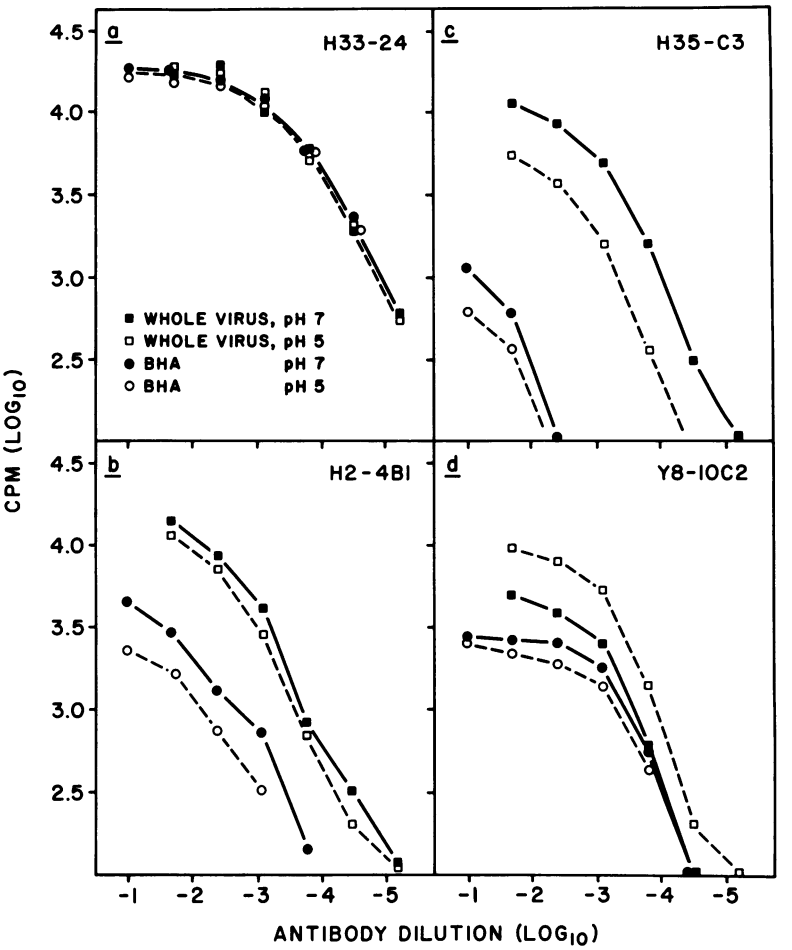

FIG. 4. Effect of pH 5 treatment on the antigenicity of whole virus and of purified HA molecules. Samples of whole virus (20 HAU) or BHA (250 ng) were allowed to adsorb to the wells of polyvinyl plastic plates. The adsorbed proteins were then incubated for 2 h at room temperature with 0.15 M sodium citrate–acetate buffer of either pH 7 or pH 5 and were tested by RIA (performed at pH 7) for their capacity to bind monoclonal antibodies. Each datum point gives the mean counts per minute observed with four replicate samples of the given antibody dilution.

virus, and antibodies to site Cb all had increased HI activity against pH 5-treated virus. Antibodies to site Ca were exceptional in that approximately one half of them had greatly increased titers and one half had greatly or slightly decreased HI titers.

The effect of pH 5-treatment of PR8 virus and of BHA molecules on the binding of anti-HA antibodies was further examined by RIA. In these experiments, intact virus or purified BHA was first adsorbed at pH 7 to wells of polyvinyl plastic plates and was subsequently treated for 2 h at 20 to 24°C with either pH 7 or pH 5 buffer. The immunoadsorbents were then washed with pH 7 buffer and compared at this pH for the binding capacity of antibodies. Figure 4 shows the titration curves of three anti-HA antibodies and one antibody (H33-24) that binds to a virus-associated carbohydrate moiety derived from the chicken host.

The overlapping titration curves of antibody H33-24 on pH 7- and pH 5-treated virus and BHA (Fig. 4a) demonstrate that the various viral immunoadsorbents were present at equivalent concentrations. Figure 4 further shows that the binding of anti-HA antibodies to pH 7- and pH 5-treated intact viral immunoadsorbent correlates roughly with the HI data (Fig. 3) in that antibodies Y8-10C2 and H35-C3 exhibited significantly increased and decreased binding, respectively, to pH 5-treated virus. In contrast to the results obtained with intact virus, this effect of pH 5 treatment on antibody binding was not seen with purified BHA; in that case all antibodies showed

decreased binding after acid treatment of the antigen. Antibody H2-4B3 (Fig. 4b), which reacted in the HI test equally well with untreated and pH 5-treated PR8 virus (Fig. 3), showed binding characteristics that were intermediate between those of antibodies Y8-10C2 and H35-C3.

Correlation between the pH dependence of viral biological activities and antigenicity. The relationship between the acid-induced alterations detected in the HI test and the decay in viral biological activities was further investigated by incubating the virus for 18 h in sodium citrate buffers of various pHs and then diluting the virus into either pH 7 buffer for the HI test and infectivity titration or into pH 5 buffer for the determination of hemolysis activity. The hemolytic activity started to decay when the virus was incubated at pH 5.4, and the extent of the decay increased with decreasing pH 5.4, (Fig. 5a). Virus infectivity was slightly more acid sensitive in that it decayed precipitously at pH below 5.6 (note that the infectivity titer is plotted on a $\log_{10}$ scale). Although the hemolysis and infectivity decay do not coincide, both occur within the pH range in which viral hemolytic activity is triggered (for a better comparison, the hemolysis activation curve of PR8 virus from Fig. 1 is replotted in Fig. 5a). This suggests that all three phenomena (acid-induced activation of hemolytic activity in the presence of erythrocytes, decay in infectivity, and hemolytic activity in the absence of erythrocytes) are manifestations of the same process.

Analysis of the acid-treated samples in HI tests is shown in Fig. 5b. It is evident that the increase in the HI titer of antibody Y8-10C2 has roughly the same pH dependence as the decay in hemolytic activity. For instance, the HI titer reached 50% of the maximum level after incubation for 18 h at pH 5.3 to 5.4, which is the same pH range at which the hemolytic activity decayed by 50%. On the other hand, the decrease in the HI titer of antibody H35-C3 paralleled the decay in viral infectivity.

The close relationship between acid-induced alteration of antigenicity and hemolytic activity was also observed when virus was treated for various periods of time with 0.15 M sodium citrate buffer (pH 5). Changes in the HI titers of antibodies Y8-10C2 and H35-C3 occurred at a rate similar to the decay of viral hemolytic activity (Fig. 6).

Relationship between HA cleavage and pH-induced antigenic alterations. Cleavage of the HA into disulfide-linked subunits has been shown to enhance viral fusion activity (8, 11, 20). It was therefore of interest to examine whether the pH-induced antigenic alterations were dependent on HA cleavage. For this purpose, PR8 virus was grown in MDCK cells. This virus, which contains mostly uncleaved HA (as verified by sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis [data not shown]), was then tested, before and after cleavage of the HA with trypsin, for the pH dependence of hemolytic activity and antigenic alteration. Trypsin treatment of MDCK-grown PR8 virus increased its hemolytic activity 4- to 10-fold, a result in agreement with the findings of Maeda et al. (12) (Fig. 7a). More importantly, however, MDCK-grown PR8 virus before and after treatment with trypsin and egg-grown PR8 virus (which contains predominantly cleaved

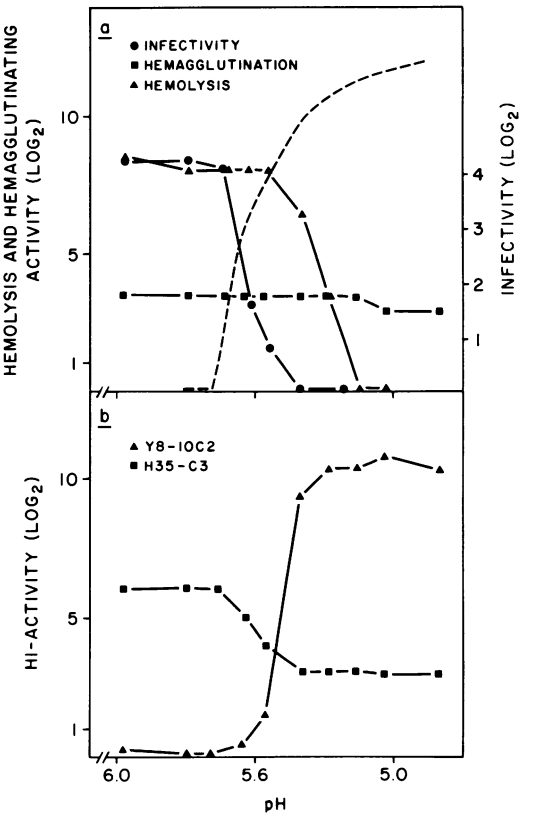

FIG. 5. Effect of pretreatment of PR8 virus at various pHs on its functional and antigenic activities. PR8 virus was incubated for 18 h at room temperature in sodium citrate buffer at the indicated pH and then was adjusted either to pH 5 for measurement of hemolysis activity or to pH 7 for determination of infectivity and hemagglutination titer against human erythrocytes (a). For ease of comparison, the pH dependence of the hemolytic activity of PR8 virus (a, stipulated curve) is replotted from Fig. 1. (b) shows the change in HI titer observed with antibodies Y8-10C2 (ascitic fluid, used at a starting dilution of 1/25) and H35-C3 (ascitic fluid, used at a starting dilution of 1/1,000) against four agglutinating doses of the various virus preparations. HI tests were performed at pH 7.

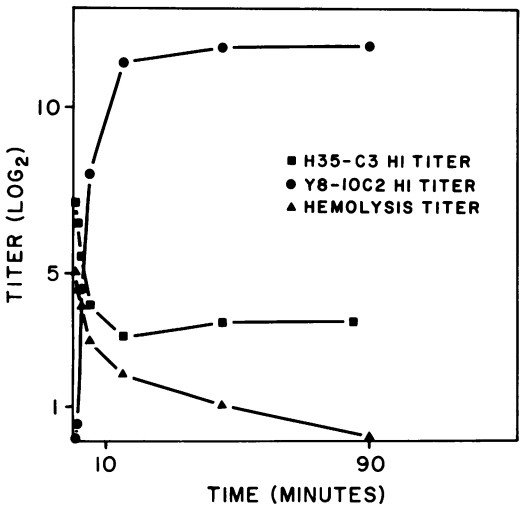

FIG. 6. Kinetics of acid-induced antigenic change. PR8 virus was incubated for various periods of time in pH 5 buffer (see Fig. 2 legend), readjusted to pH 7, and tested for reaction with antibodies Y8-10C2 and H35-C3 (see Fig. 5 legend). For comparison, the kinetics of the decay of hemolysis activity are replotted from Fig. 2.

HA) each exhibited a characteristic pH dependence of hemolysis activation, which was shifted by 0.1 to 0.2 pH unit toward the acid pH range in the following order: egg-grown, trypsin-treated MDCK-grown, and untreated MDCK-grown PR8 virus. The same general relationship was seen also in the ability of these virus preparations to interact with antibody Y8-10C2 in the HI test (Fig. 7), providing further evidence for a close relationship between the conformational changes detected by this antibody and the changes that accompany activation of hemolytic activity. Trypsin treatment per se had no detectable effect on the Y8-10C2 HI titer curve, as both trypsin-treated and untreated egg-grown PR8 virus showed identical pH dependence. Antibody Y8-10C2 also reacted to a high titer with acid-treated MDCK-grown virus despite the fact that the latter virus possesses mostly uncleaved HA (Fig. 7b). Apparently, total cleavage of the HA, though required for optimal hemolytic activity, does not seem to be a prerequisite for the acid-induced alteration detected by antibody Y8-10C2.

Ultrastructure of antibody interaction with pH 5-treated virus. The ultrastructural events accompanying the pH-induced alterations in antigenicity were followed by electron microscopy of negatively stained virus preparations before or after interaction with various hybridoma antibodies.

The morphology of viruses treated with pH 5.0 buffer was characterized by a less distinct appearance of their surface spikes and an overall deformation of the outline of the envelope membrane (Fig. 8A and B). Moreover, the penetration of stain into the core of some particles indicated further damage of the viral envelope structure. Identical structural features were noted in virus preparations incubated for up to 120 min at 37°C or up to 24 h at 25°C (data not shown). The binding of antibody Y8-10C2 to virus that was pretreated at pH 5.0 produced an easily detectable coat of proteins covering the spike molecules (Fig. 8B). Untreated virus incubated with the same antibody revealed the morphology of native influenza particles with a regular array of spikes and without the association of foreign material (Fig. 8C). In contrast, antibody H35-C3 was able to bind to virus after preincubations at pH 5.0 (data not shown) and pH 7.2 (Fig. 8D) and produced a coat which obscured the spikes. The specificity of these reactions could be demonstrated with hybridoma antibodies that are specific for matrix protein and nucleoprotein (the two major internal viral proteins), both of which failed to react with the surface spikes of acid-treated or untreated virus particles (data not shown).

From these observations, we conclude first that the acid-induced antigenic alterations detected by hybridoma antibodies are due to changes in the HA itself and are not merely a reflection of virus aggregation or disintegration, and second that the ability of antibody Y8-10C2 to inhibit hemagglutination of pH 5-treated virus but not of untreated virus results from acid-induced changes in the HA which lead either to the creation of a new epitope on the HA or to the exposure of a previously inaccessible epitope.

DISCUSSION

Skehel et al. (18) have shown that incubation of purified influenza virus HA at acidic pH, at which viral fusion activity is optimally triggered, induces an irreversible conformational transition in the HA. In the present study, we further investigated the effect of pH on antigenic, functional, and morphological properties of virus particles and solubilized HA molecules.

The antigenic analysis was performed with 44 monoclonal anti-HA antibodies. The HA structures to which these antibodies bind had been deduced previously from the failure of the antibodies to react with certain groups of PR8 virus mutants that possess defined amino acid changes in the HA1 polypeptide (2). Thus, two of the regions recognized by some of the anti-HA antibodies (and designated as antigenic sites Sa and Sb) seem to be located in close proximity to each other on the tip of each HA monomer. A third region (antigenic site Ca) seems to comprise parts of adjacent monomers in the intact

HA trimer, and a fourth region (site Cb) seems to be localized near the bottom of the globular head of the HA monomers, opposite to the monomer-monomer interface regions.

The majority of these antibodies showed significantly altered reactivity in the HI test and RIA to pH 5-pretreated virus compared with untreated virus. Since the antibody-binding assays were always performed at neutral pH, these findings indicated that an irreversible change had occurred in the virus during incubation at pH 5. This change occurred rapidly and was virtually complete after incubation of virus in pH 5 buffer for 15 min at room temperature. Also, the change seemed rather drastic because anti-HA antibodies of each specificity group (see above) were affected. However, apart from a less distinct appearance of the viral surface spikes and slight damage to the envelope structure, the change was not reflected in gross morphological changes in the viral architecture.

The observed changes in the binding capacity of anti-HA antibodies could be due either to conformational alterations within individual epitopes themselves, to alterations in antibody accessibility to the corresponding epitopes, or to both. Three observations support this second possibility: (i) isolated HA molecules did not exhibit antigenic alterations upon acid treatment; (ii) with a single exception, antibodies binding to sites Sa, Sb, and Cb showed concordant changes in binding to acid-treated virus (decreased with anti-Sa and anti-Sb antibodies and increased with anti-Cb antibodies); and (iii) antibodies that demonstrated increased binding to acid-treated virions showed similarly increased binding to HA rosettes prepared from detergent-solubilized and purified HA molecules (14). Taken together, these observations suggest that the antigenic changes in the HA may result primarily from an alteration of the spatial relationship among HA monomers within the HA trimer which, in turn, may lead to either increased or decreased accessibility to antibodies of certain sites on the HA trimers embedded in the viral envelope membrane.

With respect to viral biological activities, it was found that treatment at pH 5 led to a rapid loss in infectivity and hemolytic activity but did not alter the ability of the virus to agglutinate chicken or human erythrocytes. Therefore, the virus can apparently be transformed by acid treatment into the hemolytically active state only when it is associated with the erythrocyte membrane. It is generally accepted that the induction of hemolysis by ortho- and paramyxoviruses results from the fusion between the membranes of the viral envelope and the target cell. The ability of the influenza virus HA to promote fusion seems to depend on its prior

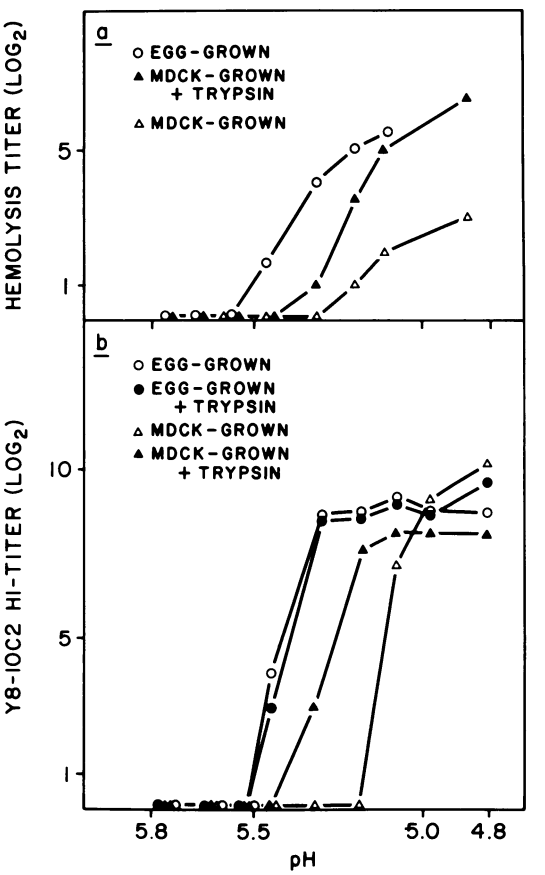

FIG. 7. Effect of cleavage of HA on pH-dependence of hemolysis activity and reactivity with antibody Y8-10C2. The pH dependence of the hemolysis activity (a) of egg-grown and MDCK-grown PR8 before and after treatment with trypsin (Trypsin TPCK; Worthington Diagnostics, Freehold, N. J.; 8 mg/ml) for 18 h at 37°C was determined as described in legend to Fig. 1. (b) shows the reaction of the same virus preparations, after adjustment to pH 7, with antibody Y8-10C2 in the HI test. In addition, egg-grown virus that had been pretreated with Trypsin TPCK (13.5 mg/ml, 2 h at 37°C) is included. HI tests were performed with ascitic fluid at a starting dilution of 1/50.

association with the cell surface (i.e., the virus receptor). The molecular events accompanying the acid fusion activation of the HA molecule could be explained in two ways. (i) The proper (hemolytic) alteration in the virus is triggered by acid solely when the virus is associated with the proper membrane structure; in the absence of this environment, acid incubation may trigger a different (nonhemolytic) alteration. (ii) The acid-triggered hemolytic state is transient, representing an intermediate state in an irreversible multistep transformation in which the subsequent product(s) possesses no hemolytic activity.

Regardless of which of the above explanations

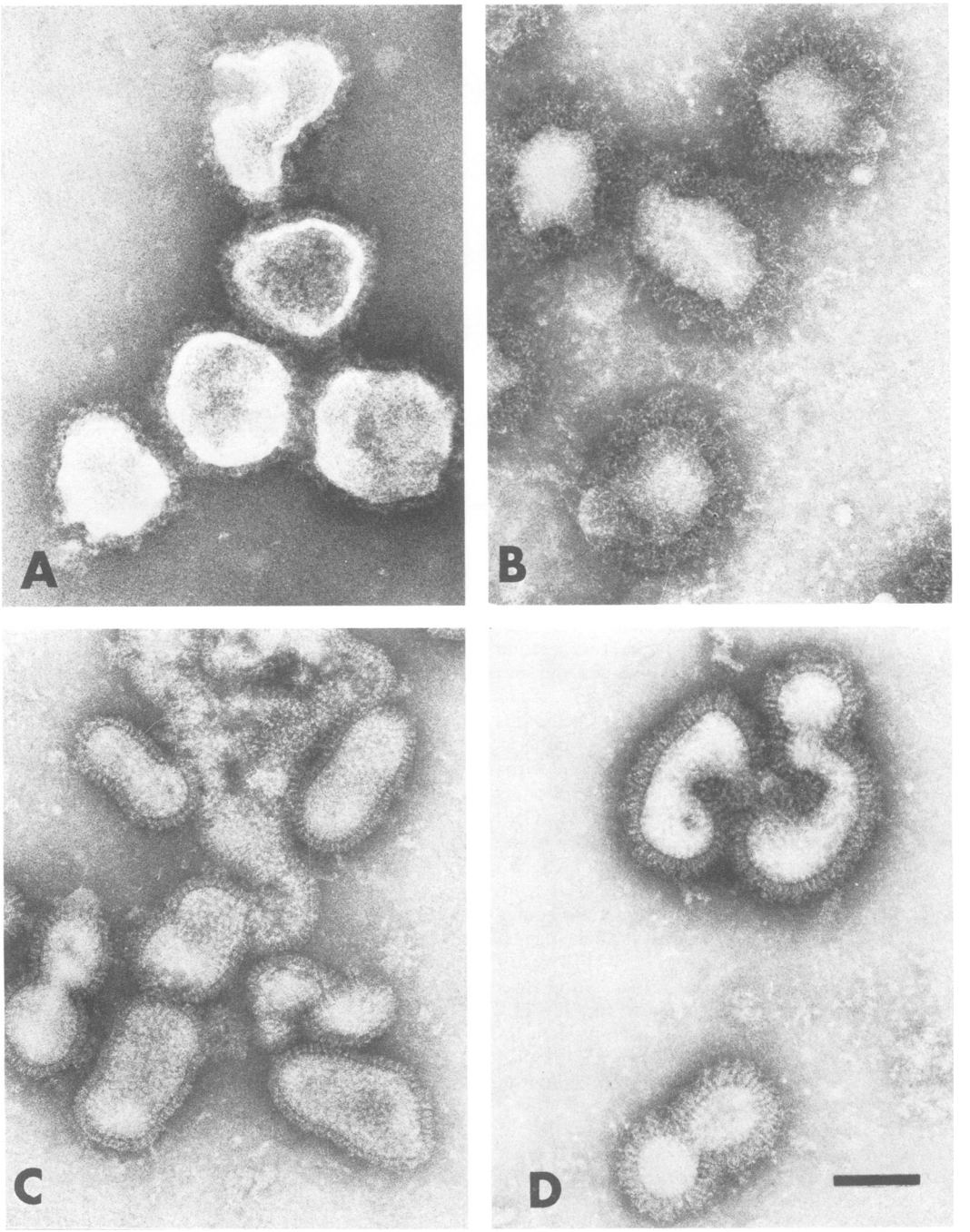

FIG. 8. Treatment of influenza virus in pH 5 buffer for 15 min at 37°C resulted in a fuzzy appearance of spikes and the penetration of stain into the particles (A). Incubation of pH 5-treated virus with antibody Y8-10C2 resulted in a thick coat of protein covering the spikes (B). Untreated virus (pH 7.2) was not reactive with this antibody and displayed surface spikes that were not associated with foreign material (C). Antibody H35-C3 reacted with virus before (D) or after (data not shown) pretreatment at pH 5.0 and obscured the spike molecules. All pictures represent negatively stained material (2% phosphotungstic acid, pH 7.0). Bar = 100 nm.

is correct, it follows that pH 5-treated virus is not identical to hemolytically active virus. This raises the question of whether the observed antigenic alterations are a direct reflection of conformational changes associated with the hemolytically active state of the HA or whether some or all of the antigenic alterations are a by-product or end product of the acid-induced triggering of hemolytic activity.

The former possibility is supported by the observation that the triggering of hemolytic activity and the induction of antigenic alterations exhibited similar pH dependencies, a relationship which was particularly striking with antibody Y8-10C2. The intimacy of hemolytic activity and Y8-10C2 activity was further demonstrated in experiments with MDCK-grown PR8 virus. In this case, both activities were found to occur at a lower pH than that for egg-grown virus and were found to shift in parallel with trypsin treatment. On the other hand, in these latter experiments we found that MDCK-grown PR8 virus, which contains mostly uncleaved HA, exhibited a degree of antigenic alterations (albeit with a different pH dependence) similar to that of egg-grown and trypsin-treated MDCK-grown virus. Because cleavage of the HA is a prerequisite for efficient hemolysis, this observation could be taken as an argument against the relevance of the antigenic alterations for the hemolytically active state of the HA. However, it is noteworthy that MDCK-grown PR8 virus, even without trypsin treatment, did produce hemolysis, although at a significantly lower efficiency than that of the egg-grown and the trypsin-treated MDCK-grown virus. Perhaps the small proportion of cleaved HA in MDCK-grown PR8 virus is sufficient to be fully detectable in the HI test but is limiting in the hemolysis assay. Also, cleavage of the HA is only one requirement for hemolysis activity; the second requirement, an acid-induced alteration, could normally occur whether HA is cleaved or not. Thus, the observation that MDCK-grown PR8 virus showed acid-induced antigenic alterations similar to those of egg-grown PR8 virus cannot be taken as an argument against the possibility that the antigenic alterations are directly related to the conformational change(s) required for the hemolytically active state.

The findings of the present study should be considered also in light of two recent reports (appearing after the original submission of this study), one by Sato et al. (17) and the other by Webster et al. (19). Our findings that hemolytic activity is rapidly lost upon acid incubation of the virus in the absence of erythrocytes and that acid treatment renders the viral envelope permeable to phosphotungstic acid stain (without disrupting the virus) are in complete agreement with the report of Sato et al. (17). There are major discrepancies, however, between the report of Webster et al. and the present study.

Webster et al. (19) used a panel of monoclonal antibodies to compare the antigenicity of untreated and pH 5-treated HA of the H3 subtype. They observed that most antibodies to the tip and interface regions (sites B and D in the H3 nomenclature [22]; see also Fig. 3) exhibited drastically (100- to 1,000-fold) reduced reactivity with pH 5-treated as compared with untreated virus, whereas antibodies to the loop region (site A) reacted equally well with untreated and pH 5-treated virus. This is in marked contrast to our observation that antibodies to sites Sa, Sb, and Ca1 (which are thought to correspond to sites B and D [2, 22]) showed, with few exceptions, only slightly (5- to 10-fold) reduced reactivity with pH 5-treated virus, whereas antibodies to site Ca2 (corresponding to site A) showed a strongly enhanced reaction with pH 5-treated virus. The antibody to the hinge region (site C) in the study done by Webster et al., which showed equal reactivity with untreated and pH 5-treated virus, cannot readily be compared with the anti-Cb (site E) antibodies in the present study, which showed a strongly increased reaction with pH 5-treated virus. Although both of the latter sites seem to be located close to the basis of the globular head of the HA monomer, they are apparently formed by different regions of the HA1 polypeptide chain (2, 22). Taken together, it is unlikely that the generally very large discrepancies between the findings made with H3 and H1 subtype HAs result from an incorrect correlation between the major antigenic regions in H3 and H1 subtype HAs or from the use in both studies of strongly biased antibody panels. Rather, these findings suggest that different antigenic regions in the H3 and H1 subtype HAs are affected differently by acid pH treatment, perhaps due to the different location of the carbohydrate side chains in these HAs. However, assuming that both H3 and H1 subtype HAs use the same mechanism for fusion and undergo the same conformational change for their activation, the discrepancies between the present study and that done by Webster et al. suggest that the observed antigenic alterations may all reflect an irrelevant by-product (end stage) of the conformational change that activates fusion.

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