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Monoclonal Antibodies Detect Different Forms of Influenza Virus Hemagglutinin During Viral Penetration and Biosynthesis

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Monoclonal antibodies specific for the influenza virus A/PR/8/34 hemagglutinin (HA) were used to examine the structure of the HA glycoprotein by immunofluorescence techniques during infection of MDCK cells. One antibody (Y8-10C2), shown previously to detect conformational alterations in the HA coinciding with the acid induction of viral fusion activity, bound to internalized virus but not to virus adsorbed to the cell surface. The binding of Y8-10C2 was completely inhibited by ammonium chloride treatment of the cells. These findings are consistent with the idea that Y8-10C2 detects conformational changes in the HA which accompany the acid-induced fusion of viral and endosomal membranes. The same antibody also bound to newly synthesized HA but not to later forms of cytoplasmic HA or to HA incorporated into the cell membrane during virus maturation. A possible common denominator of the antigenic changes detected by antibody Y8-10C2 during virus entry and replication may be alterations in the structural relationship among the three HA monomers which form the mature HA molecule.

Many enveloped viruses share a common strategy of entry into host cells which exploits the normal cellular process of adsorptive endocytosis (8, 24). Recent studies suggest that alphaviruses and orthomyxoviruses enter cells by fusing with cellular membranes after internalization and exposure to the acid conditions of the endosome (16, 17, 29, 30). Influenza A virus acquires its fusion activity (and thus infectivity) by cleavage of the hemagglutinin (HA) into disulfide-linked subunits (termed HA1 and HA2), the trimers of which form the HA spike (10, 11, 26). This cleavage occurs during biosynthesis in permissive cells (or by treatment with trypsin in vitro) and liberates the hydrophobic C-terminal region of HA2, which is thought to play an important role in the fusion process (14, 22, 27). Recent studies have further suggested that viral activity is dependent on an acid-induced conformational change in the HA (20). This conclusion is based on findings that exposure of the HA to the acid conditions which optimally induce hemolysis and fusion of viral and cellular membranes in vitro (9, 12, 15, 23, 25) also induces irreversible conformational changes in the HA (4, 20, 21, 28).

We have previously described a monoclonal anti-HA antibody (Y8-10C2) whose binding to the HA present on the intact virions coincides with conformational changes that accompany acid activation of viral fusion activity (28). Although the relevance of these changes to the fusion process is uncertain (since acid-treated virus rapidly loses its fusion activity), the binding of Y8-10C2 serves as a unique marker for the acid-induced conformational alterations in the HA. In this report, we used this antibody in immunofluorescence studies of the HA structure during viral penetration and biosynthesis, i.e., in conjunction with conditions in vivo which have not yet been amenable to demonstrations of conformational changes of the HA glycoprotein.

MATERIALS AND METHODS

Virus. Influenza virus A/PR/8/34 (H1N1) was grown in the allantoic cavity of embryonated chicken eggs. Virus used to infect Madin-Darby canine kidney (MDCK) cells at a low multiplicity of infection (MOI) was prepared by using appropriate dilutions of allantoic fluid from infected eggs. For experiments employing virus at a high MOI, virus was purified and concentrated as previously described (28). Virus titers were determined by hemagglutination assay with chicken erythrocytes and were expressed as HA units per milliliter.

Antibodies. Anti-HA hybridoma antibodies were produced and characterized as previously described (3, 5, 6). For the experiments employing immunofluorescence techniques, supernatants of hybridoma cell cultures were used in appropriate dilution with phosphate-buffered saline (PBS). An immunoglobulin G (IgG) fraction of antibody Y8-10C2 was conjugated to fluorescein isothiocyanate (FITC) as previously described (1). Rabbit anti-PR8 HA serum (no. 355) was a generous gift from J. Oxford, London. Goat anti-mouse IgG-TRITC and goat anti-rabbit IgG-TRITC were purchased from Cappel Laboratories, Cochranville, Pa.

Cells. MDCK cells were passaged by weekly trypsinizations in Dulbecco modified minimal essential Eagle medium containing 10% fetal calf serum and gentamicin. After infection of the cells with virus, medium containing 2% fetal calf serum was used. The cells used for immunofluorescence experiments were grown on cleaned glass cover slips (12 mm in diameter) placed in dishes. Cells were infected 24 to 48 h after seeding onto the glass cover slips.

Immunofluorescence. Cell monolayers were washed with cold (0°C) medium containing 2% fetal calf serum and fixed for 10 min at room temperature with a freshly prepared solution of 3% paraformaldehyde in PBS (pH 7.2). After fixation, the cells were washed with PBS. For permeabilization, fixed cells were incubated for 2 min at room temperature with a solution of 2% Triton X-100 in PBS (pH 7.2) and then washed with PBS. The immune reactions were carried out by incubating cover slips with 25 μl of an appropriate dilution of antiviral antibody followed by incubation with an anti-IgG–fluorescein conjugate. Each incubation was for 30 min at room temperature and was followed by rinsing with PBS. The stained cells were mounted on microscope slides

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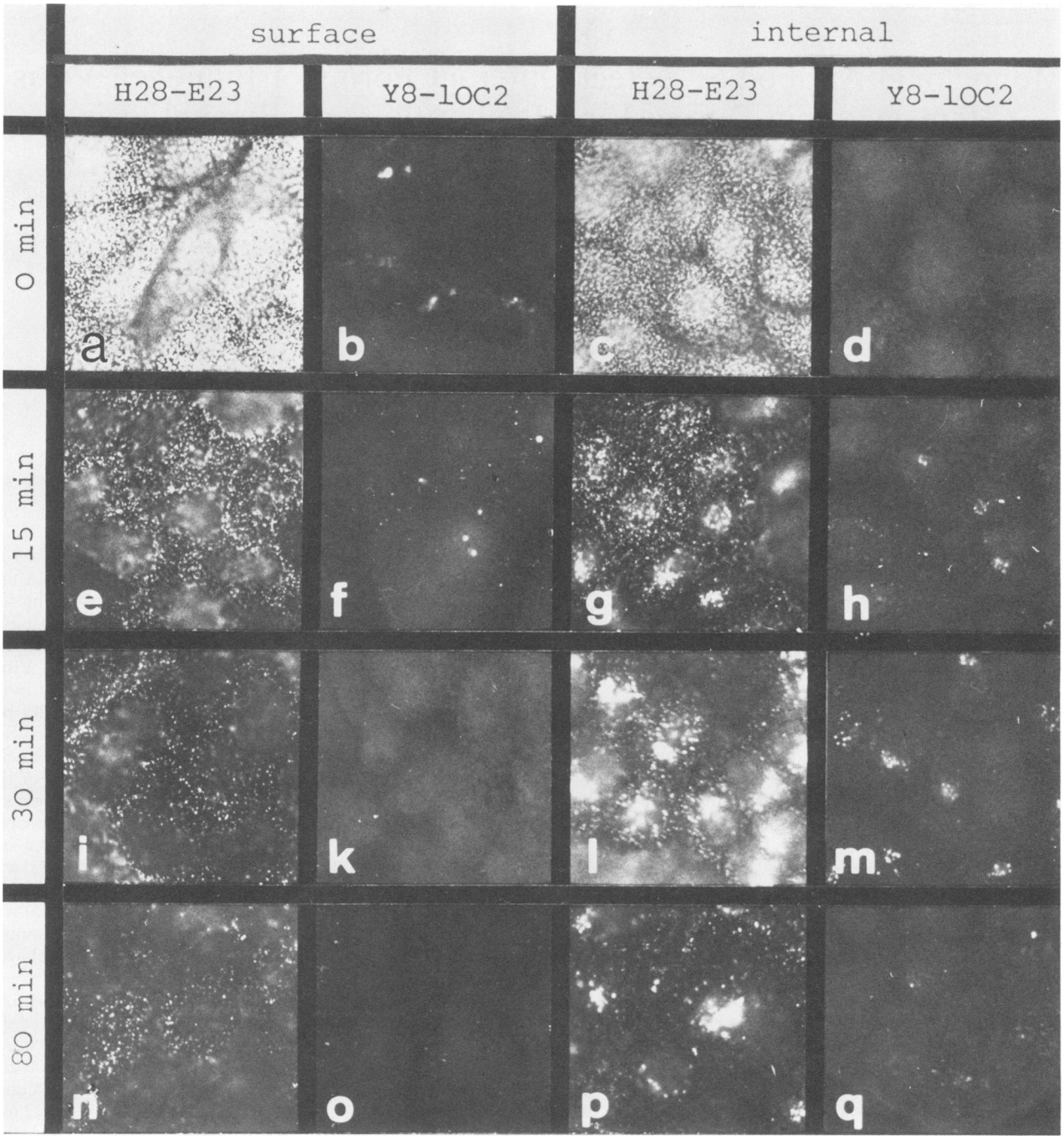

FIG. 1. Early stages of infection (0 to 80 min p.i.) of MDCK cells with influenza virus PR8. Immunofluorescence was observed with anti-HA antibody H28-E23 or Y8-10C2 after reaction with paraformaldehyde-fixed cells (surface) or fixed and permeabilized cells (internal). Prolonged incubation of cells at 37°C led to the gradual disappearance of surface-bound virus particles (a, e, i, and n) and to the temporary appearance (h and m) of fluorescent intracytoplasmic granules. Note that antibody Y8-10C2 was reactive with the HA of internalized virus (h and m) but not with that of surface-bound virus (b, d, f, k, and o).

with 50% glycerol in 0.1 M Tris hydrochloride buffer (pH 8.6). The specimens were observed with a Reichert Polyvar immunofluorescence microscope.

RESULTS

Penetration of virus into cells. To study the adsorption of virus particles onto cell surfaces and their subsequent penetration into the cells by immunofluorescence techniques, it was necessary to use a high MOI. Confluent MDCK cell monolayers on cover slips were incubated for 30 min at 0°C with 50 μl of medium containing 2% fetal calf serum and 1,000 HA units of purified virus per ml. The monolayers were then rinsed with cold medium to remove unbound virus.

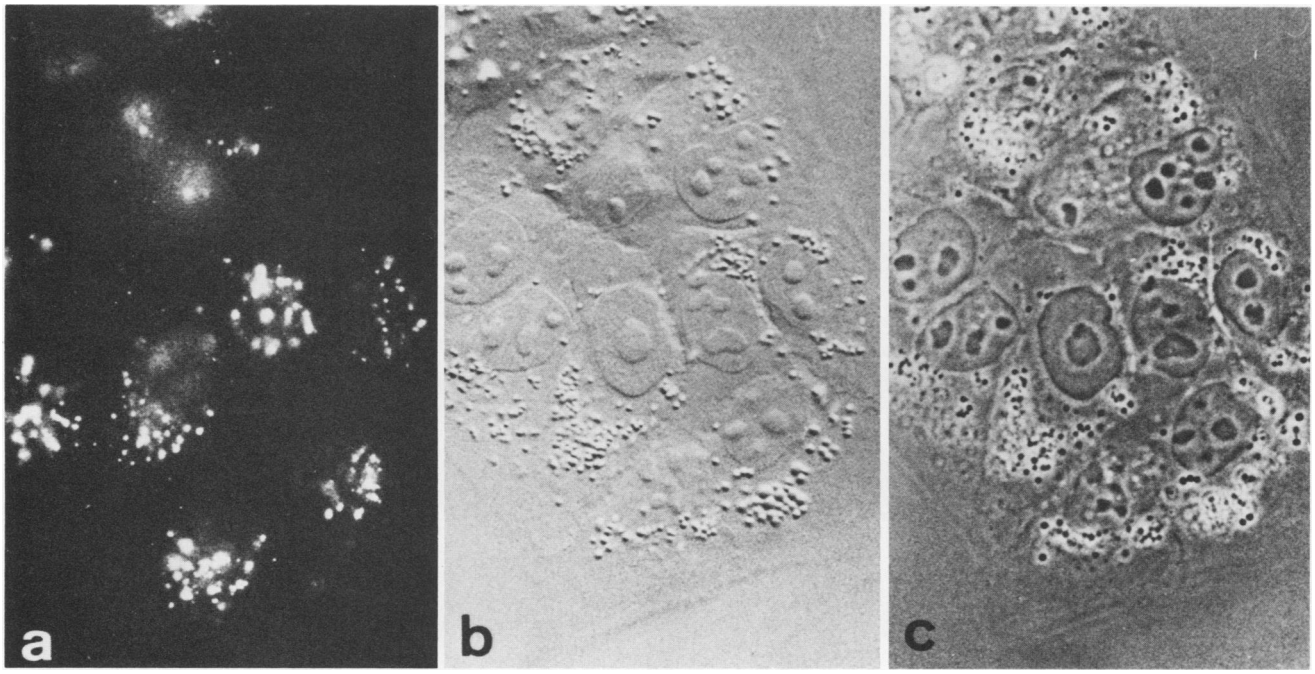

FIG. 2. Intracytoplasmic fluorescence detected with antibody Y8-10C2. Intracytoplasmic immunofluorescence observed with antibody Y8-10C2 in fixed and permeabilized PR8-infected MDCK cells 20 min p.i. (a). Corresponding field photographed by Nomarski optics (b) and phase contrast (c). The fluorescent granules do not strictly coincide with the granules seen in b and c.

Fixation of cells at the virus adsorption stage and labeling with an anti-HA antibody that binds both native and acid-treated HA (H28-E23) resulted in a granular fluorescence of the cell surface (Fig. 1a and c). In contrast, anti-HA antibody Y8-10C2 was not reactive with virus adsorbed to the cell surface (Fig. 1b and d). The infectious cycle was then allowed to proceed by the addition of prewarmed (37°C) medium to the cells. This time point is referred to below as 0 min postinfection (p.i.).

The fluorescence of surface-bound virus particles detectable with antibody H28-E23 constantly decreased from 0 to 80 min p.i. (Fig. 1a, e, i, and n). Antibody Y8-10C2 remained

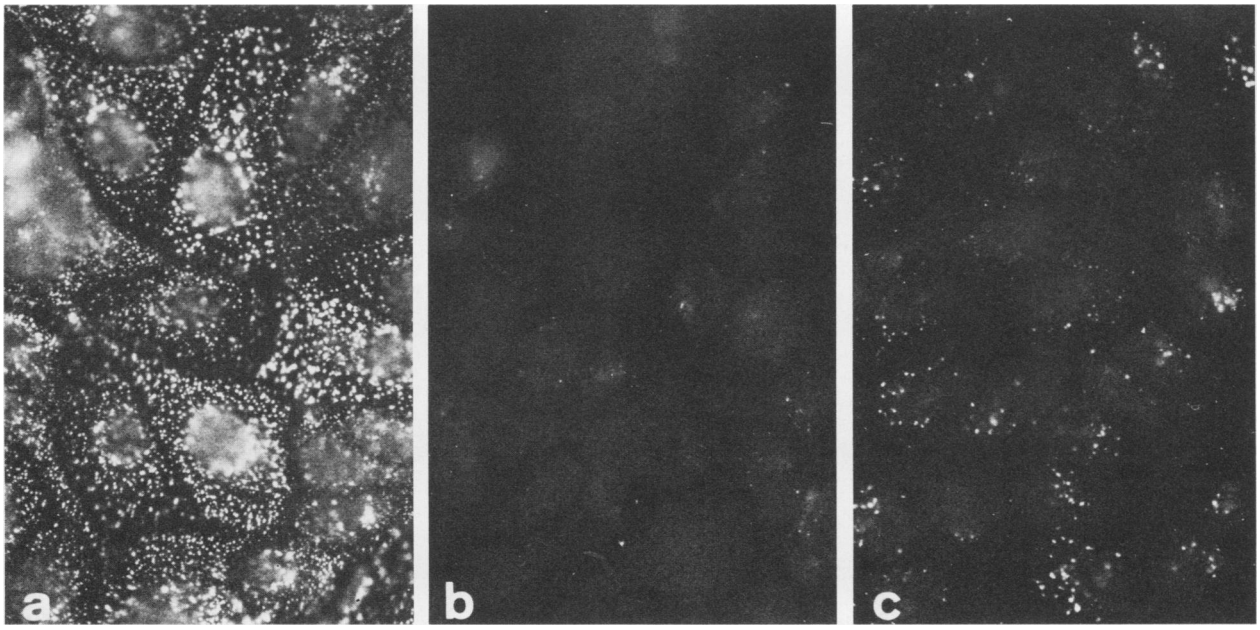

FIG. 3. Intracytoplasmic fluorescence of cells at 30 min p.i. with PR8 virus in the presence (a and b) or absence (c) of ammonium chloride detected with antibody H28-E23 (a) or antibody Y8-10C2 (b and c). Most internalized virus particles were localized in cellular compartments which were sensitive to the effect of ammonium chloride, as judged by the failure of antibody Y8-10C2 to react with HA (b), whereas internalized HA was readily detected in cells incubated without ammonium chloride (c).

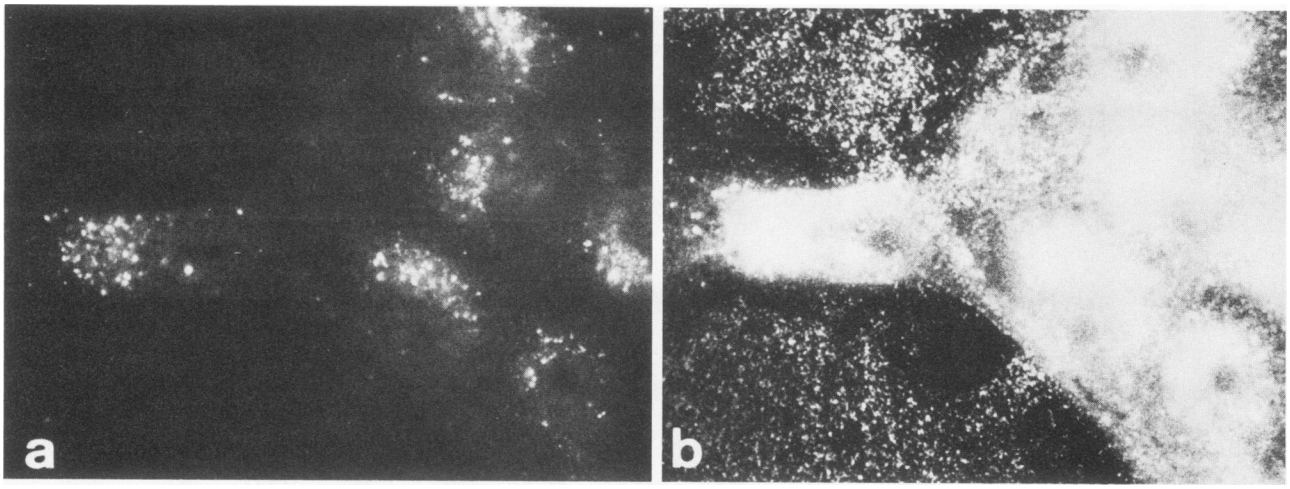

FIG. 4. Double immunofluorescence of PR8-infected MDCK cells 20 min p.i. Fixed and permeabilized cells were sequentially reacted with a rabbit anti-HA serum, a goat anti-rabbit IgG-TRITC conjugate, and an FITC conjugate of antibody Y8-10C2. The FITC fluorescence (a) detected the HA of internalized virus (see also Fig. 2), whereas the TRITC fluorescence (b) demonstrated the presence, in the same field, of virus attached to the glass surface and to cells.

unreactive with the surfaces of unpermeabilized cells at all times (Fig. 1b, f, k, and o). However, the reaction of Y8-10C2 with permeabilized cells 15 min p.i. revealed the presence of cytoplasmic antigen in the form of discrete fluorescent spots (Fig. 1h). This type of fluorescence was most prominent at 30 min p.i. (Fig. 1m and 2). A comparison of the pictures taken with fluorescence filters (Fig. 2a) with those taken by Nomarski optics (phase interference contrast) (Fig. 2b) or by phase contrast (Fig. 2c) did not reveal a direct relationship of the granules seen by the latter methods to those containing fluorescein-labeled antigen. Prolonged incubation at 37°C led to a gradual disappearance of these granules (Fig. 1q). The permeabilized cells stained with antibody H28-E23 were mainly characterized by a fine, granular fluorescence of the surface-bound antigen which did not allow the easy detection of internalized material (Fig. 1g and l). Incubation of cells in the presence of ammonium chloride (20 μmol/ml) resulted in a complete suppression of the antigenic change described above. After 30 min of incubation at 37°C, antibody H28-E23 detected both internalized and surface-bound virus particles (Fig. 3a), whereas the binding of Y8-10C2 was not detectable (Fig. 3b); cells incubated in the absence of ammonium chloride always and exclusively revealed internalized virus with antibody Y8-10C2 (Fig. 3c).

The exclusive reactivity of antibody Y8-10C2 with internalized virus was further studied by double-staining experiments. In these, the reaction of cells with an antibody Y8-10C2–FITC conjugate was followed by rabbit anti-HA and anti-rabbit IgG-TRITC. Antibody Y8-10C2–FITC stained only internalized HA (Fig. 4a), whereas the rabbit anti-HA antibody labeled intra- and extracellular virus, including virus adsorbed to the glass surface (Fig. 4b).

The influence of fixation and permeabilization procedures on the antigenicity of HA was also tested with antibody Y8-10C2. Unfixed cells and cells fixed with 3% paraformaldehyde for 1 to 80 min at room temperature and subsequently incubated for 2 to 10 min at room temperature with 0.5% Triton X-100 were compared. All of these conditions resulted in the exclusive staining of internalized virus (not shown), indicating that the binding of Y8-10C2 to internalized virus was not simply due to poor fixation of internalized antigen followed by a detergent-induced conformational change in the HA. Reaction of antibody Y8-10C2 with surface-bound virus could be artificially induced, however, by treatment of cells and virus for 5 min at 37°C with citrate buffer (pH 5.0). This resulted in a fluorescence pattern identical to the one observed with antibody H28-E23 reactive with surface-bound virus (Fig. 4b).

Biosynthesis of HA. To study the biosynthesis of HA, we infected cells at an MOI at which viral antigen could not be detected by immunofluorescence on the cell surface or in the cytoplasm at the early stages described above. With a low MOI, HA was detected as early as 90 min p.i. by both Y8-10C2 and H28-E23 as a fluorescence at the nuclear membrane and a small area surrounding the nucleus (Fig. 5a). With prolonged incubation times the fluorescence with antibody Y8-10C2 became more intense without, however, occupying more cytoplasmic space or reaching the cell surface (Fig. 5c). In contrast, the staining pattern seen with antibody H28-E23 spread into the cytoplasmic space and was superimposed by 180 min p.i. by a fine, granular fluorescence of the cell surface (Fig. 5e).

Antibody Y8-10C2 remained completely unreactive with the cell surface at all late stages of infection unless the cells were treated (at 180 min p.i.) for 5 min at 37°C with citrate buffer (pH 5.0); this resulted in a fluorescence (not shown) which was identical to the surface fluorescence of antibody H28-E23.

To further characterize the form of HA with which antibody Y8-10C2 reacted, synthesis of HA was blocked by the addition of cycloheximide (15 μg/ml) to the medium. When cycloheximide was added at 0 min p.i., the synthesis of HA was completely inhibited, as documented by the complete absence of fluorescence in such cells for up to 180 min p.i. (not shown). The addition of cycloheximide at 90 min p.i., however, allowed the synthesis of a small amount of HA before further protein synthesis was blocked. The fate of this HA was followed by further incubation of the infected cells at 37°C. The original cytoplasmic fluorescence of antibody Y8-10C2 (cf. Fig. 5a) decreased within a short time and was completely extinct after 90 min. When such cells were

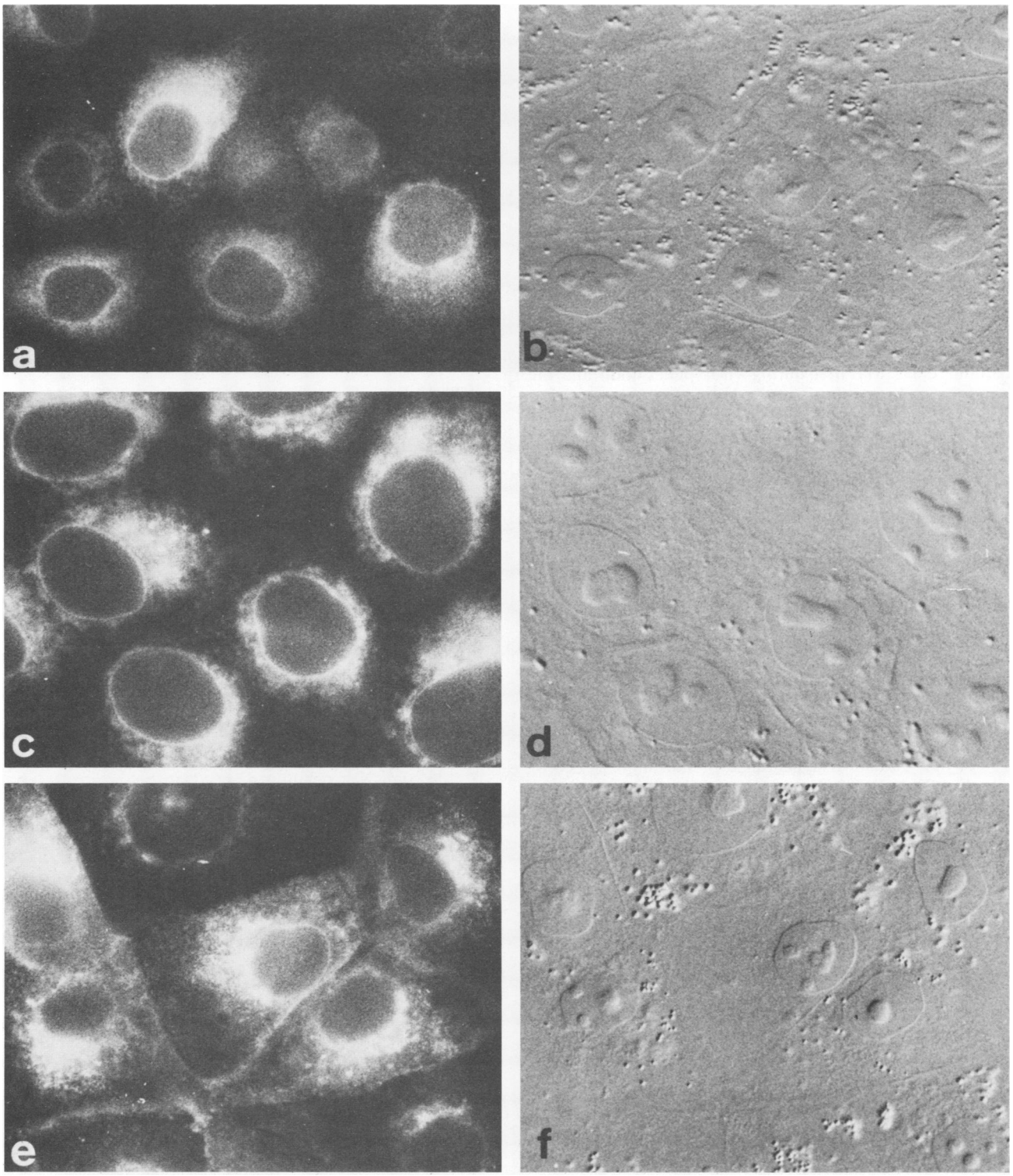

FIG. 5. MDCK cells 90 to 180 min p.i. with influenza virus. Cells were infected at a low MOI and incubated at 37°C. Immunofluorescence of permeabilized cells with antibody Y8-10C2 (a and c) or H28-E23 (e). The synthesis of HA resulted in a perinuclear staining which included the nuclear membrane and the endoplasmic reticulum at 90 min p.i. (a) and at 180 min p.i. (c and e). Antibody H28-E23 was also reactive with HA expressed at the surface and thereby created a more general fluorescence (e). Corresponding fields (b, d, and f) were photographed by Nomarski optics.

stained with antibody H28-E23, HA expressed on the cell surface was detectable, whereas all cytoplasmic HA had disappeared (not shown).

Infection of cells in the presence of tunicamycin (2 μg/ml) delayed the biosynthesis of the HA. Antibody H28-E23 detected HA on the surface of unpermeabilized cells only 5 h p.i., whereas antibody Y8-10C2 remained unreactive at this and at later stages of infection (not shown). After permeabilization of the cells, antibody Y8-10C2 was reactive with a cytoplasmic form of the HA in a pattern which was identical to the one seen in the absence of tunicamycin (Fig. 5a).

DISCUSSION

In a previous study, we showed that the anti-HA antibody Y8-10C2 recognizes an epitope that requires for its formation (or its accessibility to antibody) a conformational change in the virion or the HA molecule (28). The use of this antibody in immunofluorescence analysis revealed two conformational changes occurring in the HA in the course of the infectious cycle: the first during virus penetration and the second during biosynthesis of the HA.

The early conformational change became detectable within 15 min after the internalization of virus into the host cell. Since at this stage large amounts of virus were still present on the cell surface, detection of this alteration was possible only because antibody Y8-10C2 failed to react with surface-bound input virus. The binding of this antibody to internalized virus provides evidence that the acid-induced antigenic and conformational alteration in the HA detected in vitro (4, 20, 21, 28) also occurs in vivo as a result of the entry of the virus into the acid compartment of the cell (i.e., the endosome). Our observation that the inhibition of the infection with ammonium chloride (which is presumed to act by raising endosomal pH and preventing acid-mediated fusion) results in the decreased binding of Y8-10C2 to internalized virus supports this idea (2). The irreversible nature of the acid-induced change (28) in principle provides a marker to study the further fate of the penetrating virus particle; the gradual disappearance of the fluorescence of antibody Y8-10C2 (Fig. 1m and q) and the failure to detect the antigen on the surface of cells at any stage (Fig. 1b, f, k, and o) suggest that the bulk of infectious HA is degraded rather than recycled by the host before the onset of detectable synthesis.

The second conformational change detected by antibody Y8-10C2 occurred during the transport of newly synthesized HA to the cell surface. The perinuclear pattern of Y8-10C2 immunofluorescence suggests that Y8-10C2 detects only HA associated with the endoplasmic reticulum (7, 13, 18, 19). The addition of cycloheximide shortly after the initiation of HA biosynthesis resulted in the disappearance of perinuclear Y8-10C2-reactive HA and the appearance of Y8-10C2-nonreactive HA on the cell surface. This finding is consistent with the idea that Y8-10C2 binds to an immature form of the HA which loses its Y8-10C2 reactivity after its transport from the endoplasmic reticulum. This may mean that the conformational change detected by Y8-10C2 is related to posttranslational modifications of the HA (glycosylation, trimerization, and proteolytic cleavage) that occur during its transport to the cell surface. Since most of the HA produced during PR8 infection of MDCK cells is uncleaved, the possibility that the cleavage of the HA prevents the binding of Y8-10C2 can be safely eliminated. The inability of tunicamycin to alter the pattern of Y8-10C2 binding strongly suggests that glycosylation is not responsible for the inability of Y8-10C2 to bind the mature HA. It seems most likely, therefore, that the observed loss of reactivity of Y8-10C2 during HA biosynthesis is due to changes in the HA caused by trimerization. This possibility is attractive since, in reverse (i.e., dissociation of monomers), it could explain the binding of Y8-10C2 to acid-treated virions. In support of this idea, Daniels et al. (4) have suggested that the markedly decreased binding of a number of monoclonal anti-HA antibodies to acid-treated virus is due to the dissociation of monomer-monomer contacts in the distal region of the HA. We are currently trying to define further the relevance and structural correlate of the antigenic changes detected by antibody Y8-10C2.

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