An Efficient Method for Introducing Macromolecules into Living Cells

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ABSTRACT The hemagglutinin (HA) of influenza virus was used to obtain efficient and rapid bulk delivery of antibodies and horseradish peroxidase (HRP) into the cytoplasm of living tissue culture cells. By exploiting HA's efficient cell surface expression, its high affinity for erythrocytes, and its acid-dependent membrane fusion activity, a novel delivery method was developed. The approach is unique in that the mediator of both binding and fusion (the HA) is present on the surfaces of the target cells. A recently developed 3T3 cell line which permanently expresses HA, Madin-Darby canine kidney cells infected with influenza virus, and CV-1 cells infected with a simian virus 40 vector carrying the HA gene were used as recipient cells. Protein-loaded erythrocytes were bound to the HA on the cell surface and a brief drop in pH to 5.0 was used to trigger HA's fusion activity and hence delivery. About 3 to 8 erythrocytes fused per 3T3 and CV-1 cell, respectively, and 75–95% of the cells received IgG or HRP. Quantitative analysis showed that 1.8×10^8 molecules of HRP and 1.4×10^7 IgG molecules were delivered per CV-1 cell and 6.2×10^7 HRP molecules per 3T3 cell. Cell viability, as judged by methionine incorporation into protein and cell growth and division, was not impaired. Electron and fluorescence microscopy showed that the fused erythrocyte membranes remained as discrete domains in the cell's plasma membrane. The method is simple, reliable, and nonlytic. The ability to simultaneously and rapidly deliver impermeable substances into large numbers of cells will permit biochemical analysis of the fate and effect of a variety of delivered molecules.

With the availability of monoclonal antibodies, purified cellular proteins, effector substances, and cloned nucleic acids, it has become increasingly important to find ways to introduce such molecules into living cells for experimental purposes. To perform biochemical studies it is important to have the means to simultaneously deliver large numbers of macromolecules into most cells in a large population. To this end, a number of delivery techniques have been developed which include vehicle-mediated transfer, scrape-loading (26), and endocytosis-dependent uptake (for review see 4). Here a new method is described for bulk delivery of proteins into the cytoplasmic compartment of tissue culture cells. The technique uses loaded erythrocytes as delivery vesicles and, for attachment and membrane fusion, it exploits the activities of influenza virus hemagglutinin (HA)[1] expressed on the surface of the recipient cells. This well-characterized viral spike glycoprotein binds erythrocytes avidly by virtue of its affinity for sialic acid residues (see 33) and it can be triggered, by mildly acidic pH, to induce extensive membrane fusion (11, 21, 39–42). The recipient cells were of three different types: cells infected with influenza virus, cells infected with Simian virus 40 (SV40)

[1] *Abbreviations used in this paper*: DAB, 3,3'-diaminobenzidine; DTAF, dichlorotriazinylaminofluorescein dihydrochloride; FITC, fluorescein isothiocyanate; HA, hemagglutinin; HAO, the fusion-incompetent hemagglutinin precursor; HRP, horseradish peroxidase; MDCK, Madin-Darby canine kidney; SV40, simian virus 40; T antigen, large tumor antigen.

vectors containing the HA gene (8), and a cell line which constitutively expresses hemagglutinin (31). The procedure allows delivery to a large number of cells and the efficiency is remarkably high both in terms of the percentage of cells that receive the protein and the amount of protein delivered to each cell.

MATERIALS AND METHODS

Hemagglutinin-expressing Culture Cells: A murine 3T3 fibroblast cell line (BV1-MTHA/NIH) that expresses HA constitutively was developed by co-transfecting the cells with a bovine papilloma virus vector containing a cDNA copy of the HA gene from the influenza virus strain A/Japan/305/57 and a plasmid containing the gene for neomycin resistance (31). From this cell line we isolated a population of high HA expressors by flow cytometry as follows. Cells were grown to 80% confluency in 60-mm plates (Costar, Cambridge, MA) in Dulbecco's modified Eagles's minimal essential medium supplemented with 10 mM HEPES, 10% fetal calf serum, 250 U/ml penicillin, and 100 μg/ml streptomycin sulfate. Cells were washed in serum-free medium and incubated in trypsin (5 μg/ml) for 10 min at 22°C. The trypsin-containing medium was replaced with soybean trypsin inhibitor (25 μg/ml in PBS) for 10 min. A 1% suspension of sterile, fluorescently labeled erythrocytes (see below) in PBS was added and allowed to bind for 15 min at 22°C. Unbound erythrocytes were removed by three washes in PBS. Cells, together with erythrocytes were released from the plastic with trypsin-EDTA (0.5 g trypsin and 0.2 g EDTA in 100 ml Hank's balanced salt solution) and brought to a final concentration of 10^6 cells/ml with complete medium. After passage through a 35-μm nylon filter to remove aggregates, a subpopulation that comprised 10% of the total and exhibited the highest fluorescence intensity was separated using a fluorescence-activated cell sorter (Becton-Dickinson, Mountain View, CA) equipped with an argon laser (Spectra-Physics, Inc., Mountain View, CA) and 515- and 520-nm-long pass filters. These cells, termed the 3T3 HA-b line, were cultured as above and their HA expression was quantitated using a radioimmunoassay as described (8). For experimentation, cells were plated 2 d in advance in 35- and 60-mm culture plates (Costar), 12- and 24-well plates (Costar), or on coverslips with 12- and 18-mm diameters.

CV-1 cells, originally derived from African green monkey kidney (12), were grown in medium as described for 3T3 HA-b cells. To obtain cell surface expression of HA, we infected the cells with an SV40 late region replacement vector containing cDNA coding for HA (SVEHA3) (8). The vector, together with helper virus, was propagated in CV-1 cells as described (8), and the resulting virus stock was used to infect CV-1 cells as follows. CV-1 cells at 30–40% confluency were washed in serum-free medium and infected with an SVEHA3 stock diluted 1:10 in serum-free medium at 37°C in a 5% CO_2 incubator with gentle agitation every 15 min. After 1.5 h, the innoculum was replaced with complete medium and the cells incubated for an additional 55 h to allow HA expression.

Madin-Darby canine kidney (MDCK) cells (20) were grown in Eagle's minimal essential medium with Earle's salts containing 5% fetal calf serum and the same supplements as used for 3T3 HA-b cells. HA was expressed at the cell surface after infection with the X:31 strain of influenza virus (A/Hong Kong/1968/H3N2) (13). The virus was grown in embryonated eggs, purified as described (34), and added to monolayers for 1 h at 37°C at 10,000 HA per milliliter of serum-free medium containing 0.2% bovine serum albumin. The innoculum was replaced with complete medium and the cells were used for experimentation 16 h later.

Delivery Protocol: The hemagglutinin expressed by all three cell types described above was the fusion-incompetent hemagglutinin precursor (HAO) (8, 24, 31, 41). To activate the HAO to the mature HA, a proteolytic cleavage is necessary which generates two disulfide-linked subunits, HA_1 and HA_2 (14, 17, 41). After cells were washed with serum-free medium, the MDCK and CV-1 cells were incubated in 10 μg/ml trypsin for 10 min at 37°C and the 3T3 HA-b cells were incubated in 5 μg/ml trypsin at 22°C for 15 min. Control cells were incubated in serum-free medium without trypsin. All cells remained attached to the dishes during the treatments. MDCK and CV-1 cells were then washed in complete medium and 3T3 HA-b cells in 50 μg/ml soybean trypsin inhibitor for 5 min at 22°C. After rinsing cells in PBS, a 0.1–1% suspension of protein-loaded erythrocytes was added (usually 0.5 ml for 12- and 24-well plates and 1 ml for 35- and 60-mm plates). Erythrocytes were allowed to bind to CV-1 cells for 20 min at 22°C with gentle agitation every 5 min. Since infected MDCK cells produce the viral neuraminidase which is capable of cleaving sialic acid–containing receptors on the erythrocyte surface (3), erythrocyte binding was performed at 4°C for 20 min at 22°C with gentle agitation. Erythrocytes added to culture plates containing 3T3 HA-b cells were centrifuged onto the cells at 1000 *g* for 10 min using a Beckman J-6B centrifuge and microplate carriers (Beckman Instruments, Inc., Palo Alto, CA). After binding, the erythrocyte suspension was aspirated and "fusion medium" (PBS containing 10mM 2(*N*-morpholine)ethanesulfonic acid and 10mM HEPES, pH 5, at 37°C) was added (usually 1.5 ml/24-well plate, 2 ml/12-well plate, 3 ml/35-mm plate, and 4 ml/60-mm plate). Controls included cells expressing either HAO or mature HA exposed to medium at pH 7 or HAO-expressing cells exposed to pH 5 medium. Plates were partially immersed in a 37°C water bath for 60 s, the fusion medium was replaced by complete medium, and the plates were returned to the incubator for various periods of time. For 3T3 HA-b cells, fusion medium at pH 4.8 was used and incubation time was 5 min at 37°C. Between 30 and 60 min after fusion (or control) treatments, unfused erythrocytes were eluted from monolayers by agitation in 15–20 mg/ml neuraminidase (type V, Sigma Chemical Co., St. Louis, MO) in PBS for 30–45 min at 37°C. All other samples were agitated in complete medium. Cells were then washed 3–4 times in PBS and subjected to various experimental procedures. Some recipient cells became detached during the manipulations, but their number never exceeded 7% of the total.

Loading Erythrocytes: Fresh human erythrocytes were loaded with HRP (40 mg/ml), IgG (10–35 mg/ml), or no macromolecule (mock loaded) by the hypotonic preswell method of Rechsteiner (29). Untrapped IgG was reclaimed with 80% efficiency by high performance liquid chromatography (Gilson Medical Electronics, Inc., Middletown, WI) using a 1–330-kD gel filtration column fitted with a precolumn (both from LKB, Bromma, Sweden) and reused in subsequent loadings. Protein degradation during the loading procedure was <5% as determined by densitometric scans of autoradiograms obtained by SDS PAGE (16) of erythrocytes loaded with ^{125}I-IgG (not shown).

Purification and Iodination of IgGs: Two IgG preparations were used in delivery experiments: a monoclonal antibody directed against the large tumor (T) antigen which was produced by hybridoma cells (a gift from E. Harlow, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) grown as ascites and an IgG fraction of rabbit serum. In both cases, the IgG fraction was obtained by precipitation with ammonium sulfate as described by Unkless (36). The IgGs were further purified by affinity chromatography on either DEAE Affi-Gel Blue or DE-52 (both from Pharmacia Fine Chemicals, Piscataway, NJ) and by high performance liquid chromatography using a 1–300-kD gel filtration column fitted with a precolumn. The IgG fractions were dialysed against 10 mM Tris and then concentrated to 10 mg/ml (anti-T antigen IgG) or 35 mg/ml (rabbit IgG) in an Amicon concentrating apparatus (Amicon Corp., Danvers, MA). For quantitative experiments, 50 μg of rabbit IgG was labeled with ^{125}I by the iodogen technique (7) (1 molecule ^{125}I/4 molecules IgG) and separated from unincorporated iodine on a Dowex 50 column. After concentrating 1 ml of ^{125}I-IgG to 10 μl in a speed vacuum or by lyophilization, 1–2 × 10^7 cpms were added to 150 μl of unlabeled IgG and loaded into erythrocytes as described above. Autoradiograms from IgGs separated by SDS PAGE under reducing conditions (16) (not shown) were scanned with a densitometer and showed that IgG constituted >90% of the labeled protein in the fraction.

HRP Quantitation: We used the method of Steinman and Cohn (35) to determine the HRP activity in cell cultures after erythrocyte binding and fusion treatments. Cells were solubilized in 1% Nonidet P-40 in 10 mM Tris with aprotinin (10 μg/ml) and phenylmethylsulfonyl fluoride (~1 mM) for 1 h and assayed for HRP activity using a spectrophototmeter equipped with a strip chart recorder (Beckman Instruments, Inc.). Cells, treated in an identical manner, were trypsinized to remove them from the dishes and counted in a hemocytometer. Endogenous peroxidase activity of culture cells was negligible.

Diaminobenzidine Cytochemistry: To localize HRP at both the light and electron microscope levels we used the diaminobenzidine (DAB) method described by Graham and Karnovsky (10). Cell monolayers were fixed in 2.5% glutaraldehyde in PBS for 15 min at room temperature and incubated in 0.5 mg/ml DAB and 0.01% H_2O_2 in 50 mM Tris buffer, pH 7.6, for 45–60 min at room temperature. After washing in PBS, plates were viewed on an Olympus inverted microscope, and coverslips were mounted and examined on a Zeiss Photomicroscope equipped with phase-contrast optics.

Fluorescent Labeling of Erythrocytes: To prepare fluorescently labeled erythrocytes for cell-sorting experiments and for erythrocyte membrane protein diffusion experiments, we used the method of Fowler and Branton (6), substituting dichlorotriazinylaminofluorescein dihydrochloride for fluorescein isothiocyanate (FITC). For cell-sorting experiments, all solutions were sterilized before use. After labeling mock-loaded erythrocytes, they appeared as fluorescent spheres with peripheral rings of greater fluorescent intensity, characteristic of cell-surface staining. To more accurately determine the location of the label, we compared the fluorescent intensity of labeled erythrocytes before and after one or two cycles of lysis and resealing. Analysis by light microscopy and flow cytometry did not reveal any noticeable differences in the fluorescent pattern or intensity of lysed and intact erythrocytes, indicating that the majority of the label was located in the membrane proteins and not in content proteins.

Furthermore, the fluorescence produced by erythrocytes labeled after lysis was comparable to whole cells.

Fluorescence Microscopy: To localize IgGs by immunofluorescence, erythrocytes loaded with either rabbit IgG or monoclonal antibodies directed against T antigen were fused with HA-expressing CV-1 cells. After incubation in culture medium in a CO_2 incubator, the cells were fixed in 3% formaldehyde (wt/vol) in PBS for 15 min; the fixative was quenched in 50 mM ammonium chloride for 10 min, and the cells were permeabilized in 0.1% Nonidet P-40 for 10 min. After washing in PBS containing 0.2% gelatin, the cells were exposed to FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG for 20 min. Coverslips were then washed in PBS and mounted in moviol containing 150-mM *n*-propyl gallate to decrease photobleaching (9).

Erythrocyte spectrin was localized by indirect immunofluorescence as follows. After mock-loaded erythrocytes were fused to CV-1 cells, the preparations were neuraminidase treated and incubated for various periods of time in complete medium. Cells were cooled on ice, washed with ice-cold phosphate-buffered saline (PBS), and fixed in methanol for 10 min at −20°C. After rehydration in PBS, coverslips were first incubated in rabbit anti-spectrin IgG (20 μg/ml, a gift of J. Glenney, Salk Institute, San Diego, CA) and then in rhodamine-conjugated goat anti-rabbit IgG (a gift of M. Marsh, Yale University), and mounted. To localize erythrocyte membrane proteins, CV-1 cells were allowed to bind erythrocytes labeled with DTAF as described above. After control treatments or fusion and neuraminidase treatments, living cells were viewed with a water-immersion lens at various times after fusion. Cell surface HA was detected on formaldehyde-fixed, nonpermeabilized 3T3 HA-b and BV1-MTHA/NIH cells by indirect immunofluorescence using rabbit anti-HA serum (a gift of C. Copeland, Yale University) followed by FITC-conjugated goat anti-rabbit IgG. All preparations were examined on a Zeiss fluorescence photomicroscope equipped with an epifluorescence condenser and appropriate filters for fluorescein and rhodamine.

Transmission Electron Microscopy: Cells grown on 32-mm plates were fixed in 2.5% glutaraldehyde in 50 mM cacodylate buffer, pH 7.2, containing 50 mM KCl, 2.5 mM $MgCl_2$, and 5 mM $CaCl_2$ for 15–30 min at room temperature. For immunocytochemical demonstration of HRP, plates were incubated in the DAB solution described above for 15 min. After washing in buffer, the preparations were postfixed in 2% OsO_4 in cacodylate buffer for 15–30 min and en bloc–stained in 0.5% uranyl acetate (aqueous) overnight at 4°C. After dehydration in graded concentrations of ethanol, the monolayers were scored with a scalpel blade and sections were lifted from the plates by the solubilizing action of propylene oxide on the plastic. The sections were transferred to microcentrifuge tubes (10–15 per tube), centrifuged (4,000 *g*) into a stacked layer in a microfuge (Beckman Instruments, Inc.), and embedded in Epon 812. After reorientation of blocks for cross sectioning of monolayers, thin sections, with silver-to-gold interference colors, were cut on a Sorvall MT-2B Ultramicrotome, stained with uranyl acetate (saturated aqueous) and lead citrate and viewed in a Phillips 300 electron microscope.

Scanning Electron Microscopy: Cells grown on coverslips were fixed as above, washed in cacodylate buffer, and postfixed in 2% OsO_4 in cacodylate buffer for 30 min. They were then treated with thiocarbohydrazide and OsO_4 using a modification of the OTOTO procedure (5, 23). Cells were then dehydrated in a graded series of ethanol and critical point–dried from liquid CO_2. The specimens were coated with gold, mounted on aluminum supports, and viewed in a JEOL 100-CX electron microscope at 40 kV.

Sources of Reagents: Dulbecco's modified Eagle's medium, Eagle's minimal essential medium, and trypsin-EDTA were purchased from GIBCO Laboratories, Grand Island, NY). Aprotinin, phenylmethylsulfonyl fluoride, 3,3′-diaminobenzidine, HRP, *o*-dianisidine, trypsin (TPCK treated), soybean trypsin inhibitor, bovine serum albumin, 2(*N*-morpholino)ethansulfonic acid, tris(hydroxymethyl)aminomethane, ammonium chloride, *n*-propyl gallate, and sodium cacodylate were obtained from Sigma Chemical Co., St. Louis, MO. ^{125}I (sodium salt), and L-[^{35}S]methionine were purchased from Amersham Corp., Arlington Heights, IL. FITC-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG were from Tago Inc., Burlingame, CA; thiocarbohydrazide from Eastman Kodak Co., Rochester, NY; heparin from Hynson, Westcoff, and Dunning, Baltimore, MD, DTAF from Polysciences, Inc., Warrington, PA; Nonidet P-40 from Bethesda Research Laboratories, Gaithersburg, MD; and cycloheximide from Calbiochem-Behring Corp., San Diego, CA.

RESULTS

Erythrocyte Loading

Erythrocytes were chosen as delivery vesicles because they possess a large internal volume, because their membranes contain numerous sialic acid residues which can serve as receptors for influenza HA, and because they are easily loaded using established methods. We used the hypotonic preswell loading technique of Rechsteiner (29) which gives efficient trapping of proteins below a molecular weight of ~2×10^5. The quantitative aspects of HRP and IgG loading were analyzed in some detail. HRP, being a relatively small (40,000-mol-wt) protein, was efficiently trapped. When the initial concentration was 40 mg/ml, entrapment as measured by peroxidase activity was, on average, 2×10^7 HRP molecules per erythrocyte (40% of total). Reaction with DAB showed that 75–95% of the erythrocytes were positive. The endogenous peroxidase activity of intact erythrocytes was 1% of total, and no more than 2% of the HRP was adsorbed to the cell surface as judged by HRP attachment to unlysed erythrocytes. The amount of residual hemoglobin remaining in the erythrocytes after loading was 30–40%. The loading efficiency of ^{125}I-IgG was lower due to its larger molecular weight; at a bulk concentration of 25 mg/ml, an average of 2×10^6 IgG molecules were loaded per erythrocyte (10% of total), of which no more than 1% was adsorbed. When the loaded erythrocytes were subjected to fixation, permeabilization, and immunofluorescence using FITC-labeled anti-IgG, at least 60% were found to contain IgG.

The Target Cells

Given the strategy of our delivery procedure, only cells with influenza HA on their surface could be used as targets. Expression of HA was accomplished in three ways: infection with influenza virus (MDCK cells) (24), infection with SV40 vectors carrying the HA gene (CV-1 cells) (8), and permanent transformation of cells with HA genes (3T3 cells) (31). In each case, >95% of the cells expressed high levels of HA on their cell surfaces as judged by immunofluorescence and/or erythrocyte binding (Fig. 1 and Fig 2*a*). The hemagglutinin produced was in the form of the uncleaved precursor, HAO,

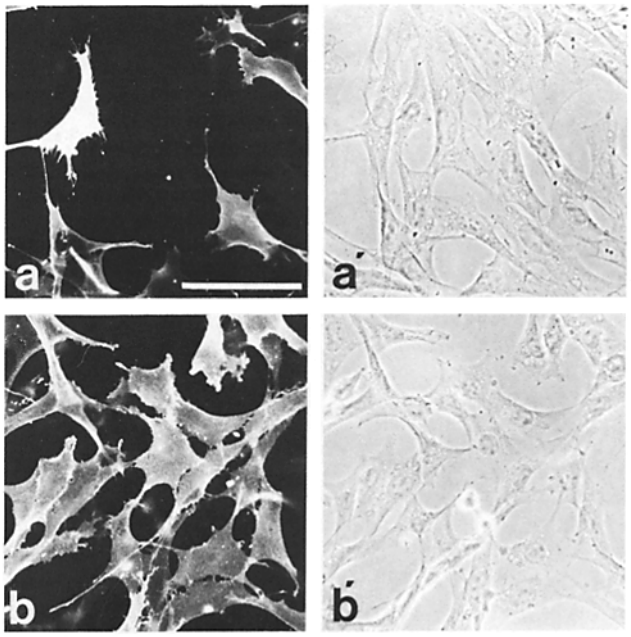

FIGURE 1 Cell surface staining for HA on 3T3 cells. BV1-MTHA/NIH cells (*a* and *a'*) and the isolated subpopulation, 3T3 HA-b cells (*b* and *b'*), were fixed and stained for HA by the indirect immunofluorescent method. *a* and *b* show the fluorescence and *a'* and *b'* the corresponding phase-contrast images. Bar, 20 μm. × 860.

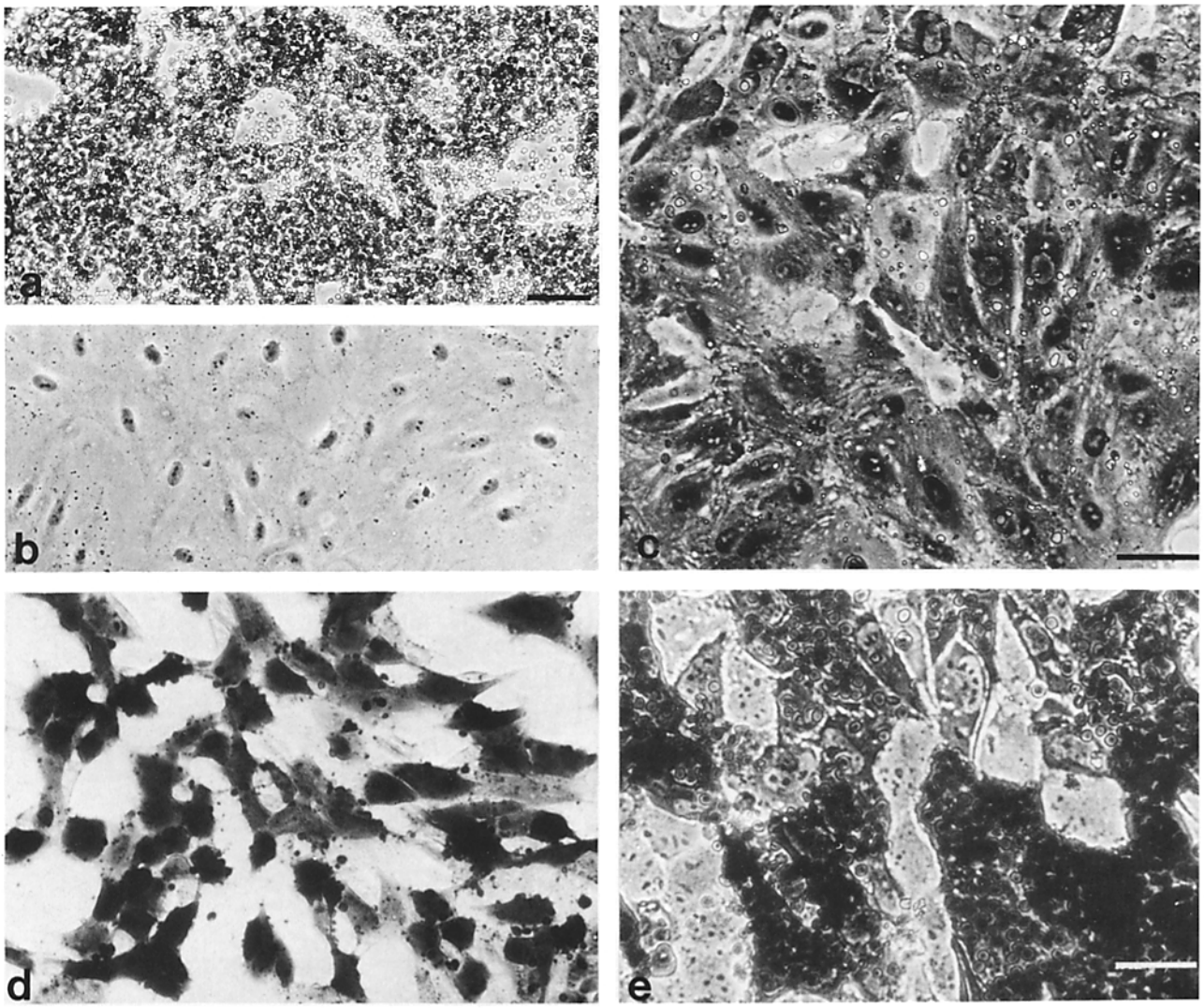

FIGURE 2 Fusion of HRP-loaded erythrocytes with different recipient cells. CV-1, 3T3 HA-b, and MDCK cells expressing either HA or the fusion-incompetent precursor, HAO, were used. After erythrocyte binding, fusion was induced by a brief acid treatment (pH 5) and unfused erythrocytes were removed by neuraminidase (where indicated). To localize the HRP, cells were fixed and reacted with DAB to produce the dark reaction product. (*a*) CV-1 cells expressing HAO (no neuraminidase). × 175. (*b*) CV-1 cells expressing HAO (with neuraminidase). × 175. (*c*) CV-1 cells expressing HA (with neuraminidase). × 225. (*d*) 3T3 HA-b cells expressing HA (no neuraminidase). × 225. (*e*) MDCK cells expressing HA (no neuraminidase). × 570. (*a–d*) Bar, 50 μm. (*e*) Bar, 20 μm.

which has full erythrocyte binding activity but lacks fusion activity (8, 11, 22, 27, 31, 39, 41). HAO was therefore activated before delivery experiments by trypsin treatment at a concentration (5–10 μg/ml) which did not release the cells from the support.

The permanently transformed 3T3 cells originated from a murine 3T3 line transformed with both a bovine papilloma virus vector containing cDNA coding for the HA gene and a plasmid coding for neomycin resistance (31). As described previously, HA expression in the parent cell line (BV1-MTHA/NIH) was variable. A subpopulation of high HA expressors was obtained by allowing the cells to bind fluorescently labeled erythrocytes and isolating the cells with the highest binding capacity on a fluorescence-activated cell sorter. The selected cell population (called 3T3 HA-b) exhibited higher and more uniform expression of HA than parent BV1-MTHA/NIH cells (Fig. 1). This type of selection has been successfully reproduced several times (Sambrook, J., unpublished results). Radioimmunoassays showed that the average number of HA molecules per 3T3 HA-b cell was 3.5 times greater than the parent line. The cells maintained their high level of HA expression for over thirty passages and thus appeared stable. Erythrocyte binding to these cells was further enhanced after trypsinization of the cells for HA activation (see above) thus increasing the final number of binding cells to 95% of the population. The obvious advantage of a permanently transformed line over the influenza- or SV40-infected cells is that they are permanently viable and require no special preparatory treatments before delivery.

Erythrocyte Binding

Attachment of erythrocytes to the various cell lines was studied morphologically (Fig. 2*a* and Fig. 3, *a* and *b*) and biochemically (Table I and II). It occurred over the entire accessible surface of cells expressing either the precursor HAO

or the mature HA (Fig 2*a* and Fig. 3, *a* and *b*) (see also references 8 and 31). On MDCK cells, numerous contacts between erythrocyte and microvilli were observed (not shown) whereas on CV-1 cells, which have few microvilli, larger flat areas of contact were seen (Fig. 3, *a* and *b*). The plasma membrane of the erythrocyte and the membrane of the recipient cell remained separated by a distance of ~20 nm. When reacted with DAB to localize the HRP, reaction product was seen only in the erythrocytes (Fig. 2*a* and Fig. 3,*a* and *b*). The binding efficiency varied depending on the cell line, time post-infection, and confluency. The conditions were optimized so that 85–95% of the cells bound erythrocytes. On average, 30 erythrocytes were associated with each MDCK and CV-1 cell with variation among individual cells. As many as 80 erythrocytes were sometimes bound to a single CV-1 cell. The 3T3 HA-b cells bound 5–10 erythrocytes each (Table I).

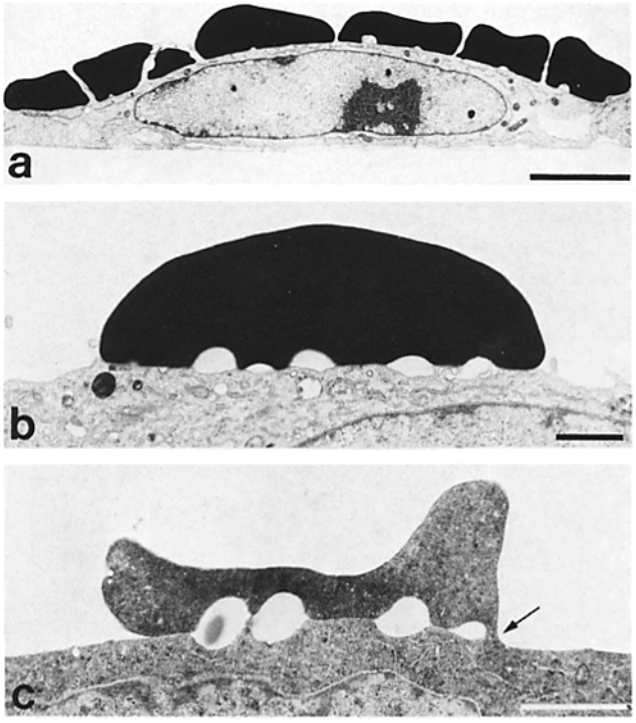

FIGURE 3 Electron microscopy of erythrocyte binding and fusion. HRP-loaded erythrocytes were bound to CV-1 cells expressing HAO (*a* and *b*) or HA (*c*). After a brief exposure to pH 5, the cells were incubated at neutral pH for 10 min, fixed, and processed for DAB cytochemistry and electron microscopy. Fusion occurred only in the cells expressing mature HA (*c*) as shown by regions of continuity between membranes (arrow) and by the presence of DAB reaction product in the target cell's cytoplasm. The fused erythrocytes frequently appeared flattened and more irregular in contour than those bound. (*a*) Bar, 5 μm; × 2,660. (*b*) Bar, 1 μm; × 8,500. (*c*) Bar, 1 μm; × 14,000.

Acid-induced Fusion and Delivery

Fusion between the erythrocytes and target cells was triggered by brief incubation in acidic medium (1 min, pH 5, unless otherwise indicated). Neutral pH was then restored, and the extent of fusion and delivery determined. The analysis was aided by the finding that unfused erythrocytes could be removed by neuraminidase. Control experiments with trypsin-activated cells kept at pH 7.0 and cells expressing the fusion-incompetent HAO showed that neuraminidase removed >95% of the bound erythrocytes (Fig. 2*b*) and 88–93% of cell-associated HRP and ^{125}I-IgG (Tables I and II).

When fusion-competent (HA-expressing) cells were used, the amount of neuraminidase-resistant HRP and ^{125}I-IgG after acid treatment was 30–40% of the total amount bound (Table I and II), suggesting that a large fraction of bound erythrocytes had fused. The increase in neuraminidase-resistant activity corresponded to the contents of three loaded erythrocytes for the 3T3 HA-b cells and eight loaded erythrocytes for the CV-1 cells. This represents at least 1.8×10^8 HRP molecules and 1.4×10^7 ^{125}I IgG molecules per CV-1 cell and 6×10^7 HRP molecules per 3T3-HA-b cell.

Light and electron microscopy after the DAB reaction showed that most of the neuraminidase-resistant HRP had been delivered into the cytoplasmic compartment of the recipient cells. At the light microscope level, 75–95% of the target cells were DAB-positive, depending on the cell type used (Fig. 2,*c–e*) and few, if any, unfused erythrocytes with reaction product could be observed after neuraminidase digestion (Fig.2*c*). Control cells that had been kept at neutral pH throughout, or cells expressing HAO, had no detectable cytoplasmic DAB reaction product (Fig. 2,*a* and *b*).

Transmission electron micrographs of CV-1 cells revealed that the membranes of many cell-associated erythrocytes had

TABLE I. *Delivery of HRP into CV-1 and 3T3 HA-b Cells**

		Erythrocyte binding		Erythrocyte fusion‡		
Cell type	Condition	HRP in bound erythrocytes (ng/5 × 10^4 cells)	Erythrocytes bound per cell	Neuraminidase-resistant HRP (ng/5 × 10^4 cells)	Neuraminidase-resistant erythrocytes per cell	HRP molecules§ delivered per cell
CV-1	HA expression pH 5 treatment	1,590 ± 109	25 ± 1.7	781 ± 63	12 ± 1.0	1.8×10^8
	HAO expression pH 5 treatment	1,628 ± 98	25 ± 1.5	195 ± 20	3 ± 0.3	—
3T3 HA-b	HA expression pH 5 treatment	449 ± 70	7 ± 1.0	247 ± 26	3.8 ± 0.4	6.2×10^7
	HA expression pH 7 treatment	456 ± 66	7 ± 1.0	46 ± 22	0.7 ± 0.3	—

* Values represent means and standard deviations of three determinations. HRP activity and cell number were determined as described in Materials and Methods. Each erythrocyte contained 2×10^7 molecules of HRP.

‡ Fusion conditions: after binding erythrocytes, the monolayers were exposed to pH 5 medium (or pH 7 medium for 3T3 HA-b control) for 1 min at 37°C, incubated in complete medium for 1 h, and treated with neuraminidase (15 mg/ml in PBS for 30–45 min at 37°C).

§ Control values subtracted.

become continuous with the plasma membrane of the recipient cells at one or more sites (Fig. 3*c*). The intensity of DAB reaction product in the erythrocytes was diminished after fusion due to the diffusion of the trapped HRP into the recipient cell cytoplasm. In contrast, the cytoplasmic compartment of target cells was more electron dense (compare to control cells, Fig. 3*c*). DAB reaction product was often observed in association with nuclei by light microscopy (Fig. 2*c*) and occasionally seen inside nuclei by electron microscopy (not shown) but was excluded from membrane-bound organelles. If the cells were fixed immediately after fusion, a gradient of reaction product could be observed, decreasing with increasing distance from the site of fusion. Fusion between the recipient cells to form polykaryons was sometimes observed, but subconfluent conditions and maximal erythrocyte binding kept the number of polykaryons to 1–2% of the total cell number.

IgG delivery was performed with two different antibody preparations: a rabbit IgG fraction and a mouse monoclonal IgG against the T antigen of SV-40. When visualized using FITC-labeled secondary antibodies, the rabbit IgG gave a diffuse staining of the cytoplasmic compartment (Fig. 4,*a–b'*). No staining was seen in the nucleus or in cytoplasmic vacuoles. In some cases, unfused erythrocytes with loaded antibody remained associated with cells after neuraminidase treatment (Fig. 4,*a*). Their presence was dependent, in part, on the time and extent of neuraminidase treatment, but the number never exceeded the background values given in Table II. When antibodies to T antigen were delivered to CV-1 cells, diffuse staining in the cytoplasm was initially seen, but upon continued incubation for 3 h, they concentrated into the nucleus (Fig. 4*c*) which is the predominant location of T antigen in SV40 infected cells (28). These results are consistent with the finding that microinjected antibodies directed against a nucleolar protein can penetrate and concentrate in nuclei relatively rapidly (1).

Taken together, the biochemical and morphological evidence clearly demonstrated that acid-dependent fusion be-

TABLE II. *Delivery of ^{125}I-IgG into CV-1 Cells**

	Erythrocyte Binding		Erythrocyte Fusion‡		
Condition	^{125}I-IgG in bound erythrocytes (cpm/6 × 10^4 cells)	Erythrocytes bound per cell	Neuraminidase-resistant ^{125}I-IgG (cpm/6 × 10^4 cells)	Neuraminidase-resistant erythrocytes per cell	^{125}I-IgG§ molecules delivered per cell
HA expression pH 5 treatment	1,762 ± 169	30 ± 3	571 ± 36	9.5 ± 0.6	1.4 ± 10^7
HAO expression pH 5 treatment	2,024 ± 211	33 ± 3	146 ± 14	2.4 ± 0.2	—

* Values represent means and standard deviations for three determinations. Cell number was determined as described in Materials and Methods. Each erythrocyte contained 2 × 10^6 molecules of IgG.
‡ Fusion and neuraminidase treatments were as described in Table I.
§ Control value subtracted.

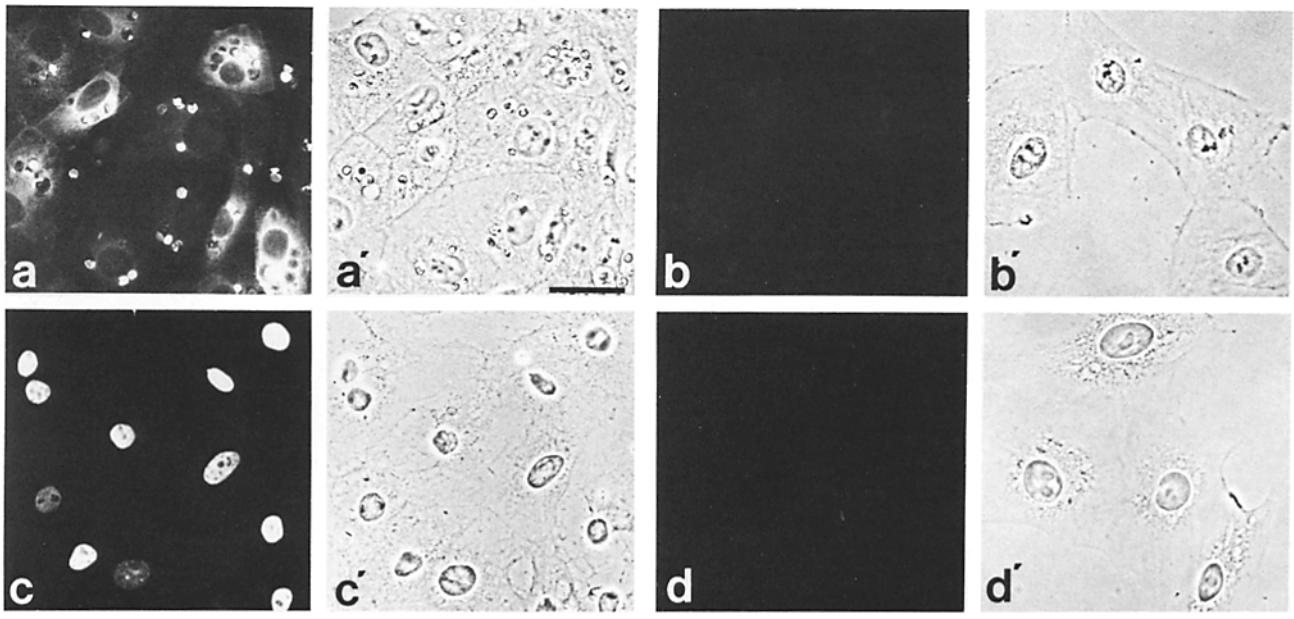

FIGURE 4 Immunofluorescence localization of IgG after delivery. Erythrocytes were loaded with either rabbit IgG or monoclonal IgG against T-antigen and allowed to bind to CV-1 cells expressing HA or HAO. Monolayers were briefly incubated in acidic medium (pH 5) and treated with neuraminidase. After a 1-h (*a–b'*) or 3-h (*c–d'*) incubation in complete medium, cells were fixed, permeabilized, and stained with the appropriate fluorescently conjugated IgG. Fluorescence (*a, b, c,* and *d*) and corresponding phase-contrast (*a', b', c',* and *d'*) images are shown. (*a* and *a'*) Cells expressing HA; reacted with rabbit IgG-loaded erythrocytes. (*b* and *b'*) Cells expressing HAO; reacted with rabbit IgG-loaded erythrocytes. (c and c') Cells expressing HA; reacted with anti-T antigen IgG-loaded erythrocytes. (*d* and *d'*) Cells expressing HAO; reacted with anti-T antigen IgG-loaded erythrocytes. Bar, 10 μm. × 1,020.

tween loaded erythrocytes and recipient cells had occurred and that it resulted in the delivery of large numbers of HRP and IgG molecules into the cytoplasm of 75–95% of the recipient cells. Even with conservative background estimates, 5×10^6 to 2×10^8 foreign protein molecules were delivered per cell.

The Fate of the Erythrocyte Membrane After Fusion

The transmission electron microscopic results suggested that erythrocytes that had fused with the culture cells remained recognizable as domains on the target cell surface (Fig. 3*c*). This was confirmed by scanning electron microscopy which showed contours of the erythrocytes on the cell surface for at least 24 h after fusion (Fig. 5*b*). To further characterize the fate of the erythrocyte membrane after fusion, we fluoresceinated the externally disposed proteins (6) prior to loading and fusion. Fig. 6 shows the appearance of CV-1 cells after DTAF-labeled erythrocytes were bound (Fig. 6*a*), or fused, neuraminidase treated, and cultured for an additional 22 h (Fig. 6*b*). Fluorescent patches of apparent erythrocyte size were clearly recognizable after this time, suggesting that the erythrocyte membrane proteins were not free to diffuse in the membrane of the recipient cell. That fusion had taken place was confirmed by HRP delivery in parallel samples using HRP-loaded, DTAF-labeled erythrocytes.

A similar result was obtained when unlabeled erythrocytes were fused to CV-1 cells and subsequently fixed, permeabilized, and stained for spectrin using anti-spectrin antibodies. For at least 22 h after fusion, the spectrin remained in discrete domains (Fig. 6*d*) not noticeably different from erythrocytes bound to the cells (Fig. 6*c*). Taken together with the fact that fusion images were detectable by electron microscopy 24 h after fusion (not shown), the results demonstrate that while the erythrocyte contents were efficiently and rapidly delivered to the recipient cell's cytoplasm, the membrane proteins and membrane skeletal components remained as discrete patches in the target cell membrane for long periods of time. Similar results for erythrocyte membrane proteins have been observed following polyethylene glycol–mediated fusion of erythrocytes with cultured cells (43).

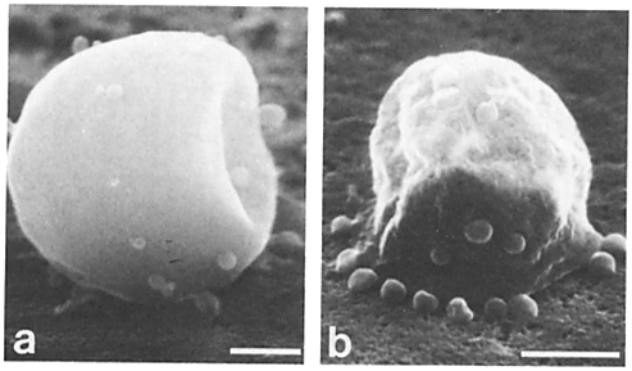

FIGURE 5 Scanning electron microscopy of erythrocyte binding and fusion. Erythrocytes (mock loaded) were bound to CV-1 cells expressing HAO (*a*) or HA (*b*) and exposed to pH 5 medium. The HA-expressing cells were neuraminidase treated to remove unfused erythrocytes. After an additional 24-h incubation in complete medium, the cells were processed as described. Similar results were obtained when neuraminidase treatment was omitted. (a) Bar, 1 μm. × 10,000. (*b*) Bar, 1 μm. × 14,600.

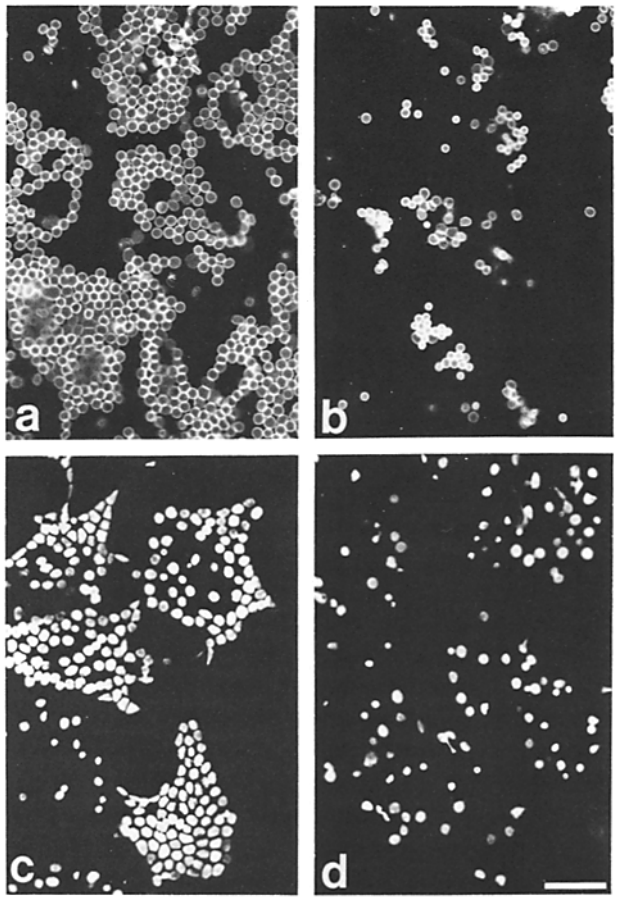

FIGURE 6 Fate of erythrocyte membrane proteins and spectrin after binding and fusion. Erythrocytes that had fluorescently labeled membrane proteins were bound to CV-1 cells expressing either HAO (*a*) or HA (*b*). The cells were briefly incubated in acidic medium. The HA-expressing cells (*b*) were treated with neuraminidase to remove unfused erythrocytes. Living cells were viewed 22 h later. To localize spectrin (*c* and *d*), erythrocytes were bound to CV-1 cells expressing HAO (*c*) or HA (*d*) and treated as above. 22 h later, cells were fixed, permeabilized, and stained for spectrin by the indirect immunofluorescence method. Bar, 10 μm. × 920.

Cell Viability

To determine whether the delivery protocol had deleterious effects on the recipient cells, we monitored cell growth and division of 3T3 HA-b cells, and the incorporation of [^{35}S]-methionine in CV-1 cells. As shown in Tables III and IV, the results indicate that trypsinization, low pH treatment, implantation of foreign membrane, and delivery of foreign protein had no detectable effects on cell viability and protein synthesis. Bear in mind that the CV-1 cells were reaching the lytic stage of the SV40 infection by 24 h after fusion. However, the 3T3 HA-b cells remained viable and could be passaged in culture for several weeks after fusion.

DISCUSSION

Loaded erythrocytes and viral fusogens have previously been used in numerous delivery strategies (for review see reference 32). The technique described here is unique in two respects: it exploits for the first time an efficient, acid-triggered viral fusion protein (the influenza HA), and it takes advantage of recipient cells that synthesize and express this protein on their surface. The need for preparing virus or reconstituted vesicles

TABLE III. *Effects of Erythrocyte Fusion on Viability of 3T3 HA-b Cells**

Conditions	Cell number × 10^{4}‡		
	0 h	*24 h*	*48 h*
pH 5 treatment with erythrocytes	2.5	3.2	7.0
pH 5 treatment without erythrocytes	2.5	2.9	6.8
pH 7 treatment without erythrocytes	2.5	3.6	6.7

* Cells in 60-mm dishes were trypsinized to activate the hemagglutinin, allowed to bind erythrocytes, and exposed to pH 5 medium to trigger fusion. 1 h later, cells were removed from the dishes with trypsin-EDTA and equal numbers from each group (2.5 × 10^{4}) were plated in 35-mm plates. At 24 and 48 h, the cells were removed from the plates and counted.
‡ Average of two determinations.

TABLE IV. *Effects of Erythrocyte Fusion on Viability of CV-1 Cells as Measured by [^{35}S]Methionine Incorporation*

Conditions	Incorporation of [^{35}S]methionine per well (cpm × 10^{-3})		
	1 h	*6 h*	*22 h*
HA, pH 5, + erythrocytes	35 ± 4.2	22 ± 5.2	22 ± 2.6
HA, pH 5, − erythrocytes	37 ± 3.0	23 ± 2.1	23 ± 2.3
HA, pH 7, + erythrocytes	41 ± 4.1	24 ± 0.9	21 ± 1.1
HAO, pH 7, − erythrocytes	25 ± 0.8	22 ± 2.4	25 ± 3.0

CV-1 cells were used 50 h after infection with the SV40 vector (SVEHA3). At this time, 80% bound erythrocytes. The cells were trypsinized to activate the hemagglutinin and allowed to bind mock-loaded erythrocytes. After brief exposure to acid conditions (pH 5) to trigger fusion, cells were incubated in complete medium for the times indicated. Various control conditions were used. Cells were assayed for the incorporation of [^{35}S]methionine using a 10-min pulse and 15-min chase as previously described (39) except that after the chase period, cells were washed three times with PBS with 10 mM methionine at 22°C and solubilized immediately. Values from cycloheximide-treated cells (1 μg/ml) have been subtracted. Values are means and standard deviations for four determinations.

is eliminated, no exogenously added agglutinins or fusogens are required, and no special equipment is used. The HA molecules in the plasma membrane of the recipient cells serve a dual function: they bind the loaded erythrocytes by virtue of their lectin specificity for sialic acid, and they mediate fusion when briefly exposed to low pH. The resultant delivery is highly efficient, reproducible, and nondeleterious to the cells. Simultaneous delivery to many culture cells is possible and nearly all cells in the population receive large numbers of test molecules.

Our results, using two proteins, demonstrate that the method has several advantages over alternative bulk procedures for protein delivery. Such methods include fusion of loaded liposomes or erythrocytes using Sendai virus or polyethylene glycol, reconstituted vesicles containing viral fusion proteins, mechanical rupture of the plasma membrane by scraping (26), hypotonic swelling (2), and osmotic lysis of pinocytic vesicles (for review see reference 4). On the whole, the present method is more efficient, less damaging, and more easily controlled.

However, this method is restricted to cells that express large numbers of HA molecules on their surface. Sufficient expression is obtainable by infection with influenza virus, which has a broad host range, with SV40 vectors containing the HA genes or by permanent transformation using other vector systems. When infected cells are used, experimentation is limited to a period of ~24 h, and limited to questions concerning cellular functions that are unaffected by the viral infection. Many cellular processes, including protein synthesis, membrane transport, and endocytosis, continue late into infection by SV40 and influenza, but other functions may be modified.

The use of permanently transformed cell lines, such as the murine 3T3 HA-b line, circumvents many of these problems. Since synthesis of the HA need not be induced by infection before experimentation, the 3T3 HA-b cells are simple to use and the efficiency of delivery, although still lower than that in the SV40-infected CV-1 cells, is sufficient for most applications. It is hoped that these cells will become useful as universal recipient cells into which a variety of molecules and reagents can be delivered using single and multiple fusions. The cells may thus serve as living "test tubes" for the study of interactions between delivered and cellular molecules under cytoplasmic conditions as well as between delivered molecules themselves. Although at present the number of HA-expressing cell types is limited, others are being developed (Hunter, E., and J. Sambrook, unpublished results), and the potential exists for the production of cell lines from a variety of species.

Previous studies have shown that antibodies introduced into the cytoplasm of culture cells remain stable for several days (25) and are capable of recognizing and inactivating their antigens (for review see reference 30; also see 15, 18, 19). We are presently using the method described here to study intracellular membrane traffic by delivering antibodies directed against proteins located on the cytoplasmic aspect of components of the vacuolar apparatus. Although developed for protein delivery, the technique is potentially useful for other classes of molecules ranging from ions to nucleic acids. We are now developing methods for delivery of nucleic acids, using either erythrocytes (32) or liposomes (see reference 4) as carriers. Ganglioside-containing liposomes have already been successfully used for implantation of foreign lipids into the plasma membrane of influenza-infected MDCK cells (37, 38).

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REFERENCES

1. Bennett, F. C., H. Busch, M. A. Lischwe, and L. C. Yeoman. 1983. Antibodies to a nucleolar protein are localized in the nucleolus after red blood cell–mediated microinjection. *J. Cell Biol.* 97:1566–1572.
2. Borle, A. B., and K. W. Snowdowne. 1982. Measurement of intracellular free calcium in monkey kidney cells with aquorin. *Science (Wash. DC).* 217:252–254.
3. Bucher, D., and P. Palese. 1975. The biologically active proteins of influenza virus: neuraminidase. *In* The Influenza Viruses and Influenza. E. D. Kilbourne, editor. Academic Press, Inc., New York. 83–123.
4. Celis, J. E. 1984. Microinjection of somatic cells with micropipettes: comparison with other transfer techniques. *Biochem. J.* 223:281–291.
5. Connolly, J. L., S. A. Green, and L. A. Greene. 1984. Comparison of rapid changes in surface morphology and coated pit formation of PC12 cells in response to nerve growth factor, epidermal growth factor, and dibutyryl cyclic AMP. *J. Cell Biol.* 98:457–465.
6. Fowler, V., and D. Branton. 1977. Lateral mobility of human erythrocyte integral membrane proteins. *Nature (Lond.).* 268:23–26.
7. Fraker, P. J., and J. C. Speck, Jr. 1978. Protein and cell membrane iodinations with a sparingly soluble 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80:849–857.
8. Gething, M.-J., and J. Sambrook. 1981. Cell-surface expression of influenza haemagglutinin from a cloned DNA copy of the RNA gene. *Nature (Lond.).* 293:620–625.
9. Giloh, H., and J. W. Sedat. 1982. Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by *n*-propyl gallate. *Science (Wash. DC).*

217:1252–1255.
10. Graham, R. C., Jr., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291–302.
11. Huang, R. T. C., R. Rott, and H.-D. Klenk. 1981. Influenza viruses cause hemolysis and fusion of cells. *Virology.* 110:243–247.
12. Jensen, F. C. 1964. Infection of human and simian tissue cultures with Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 52:53–59.
13. Kilbourne, E. D. 1969. Future influenza vaccines and the use of genetic recombinants. *Bull. W. H. O.* 41:643–645.
14. Klenk, H.-D., R. Rott, M. Orlich, and J. Blodorn. 1975. Activation of influenza A viruses by trypsin treatment. *Virology.* 68:426–439.
15. Klymkowsky, M. W., R. H. Miller, and E. B. Lane. 1983. Morphology, behavior, and interaction of cultured epithelial cells after the antibody-induced disruption of keratin filament organization. *J. Cell Biol.* 96:494–509.
16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–684.
17. Lazarowitz, S., and P. Choppin. 1975. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. *Virology* 68:440–454.
18. Lin, J. J.-C., and J. R. Feramisco. 1981. Disruption of the in vivo distribution of the intermediate filaments in fibroblasts through the microinjection of a specific antibody. *Cell* 24:185–193.
19. Mabuchi, I., and M. Okuno. 1977. The effect of myosin antibody on the division of starfish blastomeres. *J. Cell Biol.* 74:251–263.
20. Madin, S. H., and N. B. Darby, Jr. 1958. In American Type Culture Collection Catologue of Strains II. Rockville, MD. 52. 1981.
21. Haeda, M., and S. Ohnishi. 1980. Activation of influenza virus by acidic media causes hemolysis and fusion of erythrocytes. *FEBS (Fed. Eur. Biochem. Soc.)* Lett. 122:283–287.
22. Maeda, T., K. Kawasaki, and S. Ohnishi. 1981. Intraction of influenza virus haemagglutinin with target membrane lipids is a key step in virus-induced hemolysis and fusion at pH 5.2. *Proc. Natl. Acad. Sci. USA.* 78:4133–4237.
23. Malick, L. E., R. B. Wilson, and D. Stetson. 1975. Modified thiocarbohydrazide procedure for scanning electron microscopy: routine use for normal, pathological or experimental tissues. *Stain Technol.* 50:265–269.
24. Matlin, K. S., and K. Simons. 1983. Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell.* 34:233–243.
25. McGarry, T., R. Hough, S. Rogers, and M. Rechsteiner. 1983. Intracellular distribution and degradation of immunoglobulin G and immunoglobulin G fragments injected into HeLa cells. *J. Cell Biol.* 96:338–346.
26. McNeil, P. L., R. F. Murphy, F. L. Lanni, and D. L. Taylor. 1984. A method for incorporating macromolecules into adherent cells. *J. Cell Biol.* 98:1556–1564.
27. Ohuchi, M., R. Ohuchi, and K. Mifune. 1982. Demonstration of hemolytic and fusion activities of influenza C virus. *J. Virol.* 42:1076–1079.
28. Pope, J. H., and W. P. Rowe. 1964. Detection of specific antigen in SV40-transformed cells by immunofluorescence. *J. Exp. Med.* 120:121–128.
29. Rechsteiner, M. C. 1982. Transfer of macromolecules using erythrocyte ghosts. *In* Techniques in Somatic Cell Genetics. J. Shay, editor. Plenum Publishing Corp., New York. 385–398.
30. Rechsteiner, M., and R. Schlegel. 1985. Erythrocyte-mediated transfer: applications. *In* Microinjection and Organelle Transplantation Techniques: Methods and Applications. J. E. Celis, A. Graessman, and A. Loyter, editors. Academic Press Inc., New York.
31. Sambrook, J., L. Rogers, J. White, and M.-J. Gething. 1985. Lines of murine cells that constitutively express influenza virus hemagglutinin. *EMBO (Eur. Mol. Biol. Organ. J.)* 4:91–103.
32. Schlegel, R. A., and M. C. Rechsteiner. 1985. Erythrocyte-mediated transfer: methods. *In* Microinjection and Organelle Transplantation Techniques: Methods and Applications. J. E. Celis, A. Graessman, and A. Loyter, editors. Academic Press, Inc., New York.
33. Schulze, I. T. 1975. The biologically active proteins of influenza virus: the hemagglutinin. *In* The Influenza Viruses and Influenza. E. D. Kilbourne, editor. Academic Press, Inc., New York. 53–82.
34. Skehel, J. J., and G. C. Schild. 1971. The polypeptide composition of influenza A viruses. *Virology.* 44:396–408.
35. Steinman, R. M., and Z. A. Cohn. 1972. The interaction of soluble horseradish peroxidase with mouse peritoneal macrphages in vitro. *J. Cell Biol.* 55:186–204.
36. Unkless, J. C. 1979. Characterization of a monoclonal antibody against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med.* 150:580–596.
37. Van Meer, G., and K. Simons. 1983. An efficient method for introducing defined lipids into the plasma membrane of mammalian cells. *J. Cell Biol.* 97:1365–1374.
38. Van Meer, G., J. Davoust, and K. Simons. 1985. Parameters affecting low pH-mediated fusion of liposomes with the plasma membrane of cells infected with influenza virus. *Biochemistry.* In press.
39. White, J., K. Matlin, and A. Helenius. 1981. Cell fusion by Semliki forest, influenza, and vesicular stomatitis viruses. *J. Cell Biol.* 89:674–679.
40. White, J., A. Helenius, and J. Kartenbeck. 1982. Membrane fusion activity of influenza. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:217–222.
41. White, J., A. Helenius, and M.-J. Gething. 1982. Haemagglutinin of influenza expressed from a cloned gene promotes membrane fusion. *Nature (Lond.).* 300:658–659.
42. White, J., M. Kielian, and A. Helenius. 1983. Membrane fusion proteins of enveloped animal viruses. *Q. Rev. Biophys.* 16:151–195.
43. Wojcieszyn, J. W., R. A. Schlegel, K. Lumley-Sapanski, and K. A. Jacobson. 1983. Studies on the mechanism of polyethylene glycol–mediated cell fusion using fluorescent membrane and cytoplasmic probes. *J. Cell Biol.* 96:151–159.