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 x 10^8 molecules of HRP and 1.4 x 10^7 IgG molecules were delivered per CV-1 cell and 6.2 x 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) 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)

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 in both of the parameters 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 (BVI-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 Eagle’s minimal essential medium supplemented with 10 mM HEPES, 10% fetal calf serum, 250 µM 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 30 min at 22°C. Unbound erythrocytes were removed three times with PBS, and the cells, together with attached 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 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 10 mM 2-N-morpholinoethanesulfonic acid and 10 mM 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 plate 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 took place at 37°C for 5 min. Between 30 and 60 min after fusion (or control) treatments, unfused erythrocytes were eluted from monolayers by agitating in 15–20 mg/ml neuraminidase (type V, Sigma Chemical Co., St. Louis, MO) in PBS for 10–30 min at 37°C. Cells 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 prestaining method of Rechsteiner (29). Unstained IgG was reconstituted with 80% efficiency by high performance liquid chromatography (Gilson Medical Electronics, Inc., Middleton, WI) using a 1–330-kD gel filtration column fitted with a precolumn (both from LKB, Bromma, Sweden) and used 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 125I-IgG (not shown).

**Purification and Iodination of IgGs:** Two IgG preparations were used: monoclonal antibodies to a polyclonal antibody to a 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 an 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 125I by the iodogen technique (37) (1 molecule 125I/mole IgG) and separated from unincorporated iodine on a Dowex 50 column. After concentrating 1 ml of 125I-IgG to 10 µl in a speed vacuum or by lyophilization, I–2 x 105 cpm were added to 100 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 fractions.

**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 spectrophotometer 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 microscroe levels we used the diaminobenzidine (DAB) method described by Graham and Karnovsky (10). Cell monolayers were fixed in 2.5% glutaraldehyde in PBS for 20 min at room temperature and incubated in 0.05 mg/ml DAB and 0.01% H2O2 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 removed 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 by filtration. After labeling monoclonal antibodies, they appeared as fluorescent crescentic 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.

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(Contains references and further details related to the methods and procedures used in the research, including the use of specific reagents and techniques for cell manipulation and dye labeling.)
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 CO2 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 mowiol containing 150-mM n-propyl gallate to decrease photo-bleaching.

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 BVI-MTHA/NIH cells 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 x 10^5 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 x 10^4 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 2a). The hemagglutinin produced was in the form of the uncleaved precursor, HA0,
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. 2a 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 2a 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. 2a 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).

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 HA showed that neuraminidase removed >95% of the bound erythrocytes (Fig. 2b) and 88–93% of cell-associated HRP and 125I-IgG (Tables I and II).

When fusion-competent (HA-expressing) cells were used, the amount of neuraminidase-resistant HRP and 125I-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 $1.8 \times 10^5$ HRP molecules and $1.4 \times 10^7$ 125I-IgG molecules per CV-1 cell and $6 \times 10^7$ 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. 2c–e) and few, if any, unfused erythrocytes with reaction product could be observed after neuraminidase digestion (Fig. 2c). Control cells that had been kept at neutral pH throughout, or cells expressing HAO, had no detectable cytoplasmic DAB reaction product (Fig. 2a and b).

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

![Figure 3](image-url) - 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.

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

| Cell type | Condition | HRP in bound erythrocytes (ng/5 x 10^4 cells) | Erythrocytes bound per cell | Neuraminidase-resistant HRP (ng/5 x 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 x 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 x 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 x 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. 3c). 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. 3c). DAB reaction product was often observed in association with nuclei by light microscopy (Fig. 2c) 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. 4a–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. 4a). 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. 4c) 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 125I-IgG into CV-1 Cells**

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

*Values represent means and standard deviations for three determinations. Cell number was determined as described in Materials and Methods. Each erythrocyte contained 2 x 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. x 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, \(5 \times 10^6\) to \(2 \times 10^8\) 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. 3c). 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. 5b). 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. 6a), or fused, neuraminidase treated, and cultured for an additional 22 h (Fig. 6b). 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. 6d) not noticeably different from erythrocytes bound to the cells (Fig. 6c). 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 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 \(\mu\)m. \(\times\) 920.

**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...
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 nondestructive 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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