

“ULTRAMICROINJECTION” OF MACROMOLECULES OR SMALL PARTICLES INTO ANIMAL CELLS

A New Technique Based on Virus-Induced Cell Fusion

ABRAHAM LOYTER, NEHAMA ZAKAI, and RICHARD G. KULKA

From the Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

ABSTRACT

A new method is described for the introduction of macromolecules and small particles into animal cells. The first step in this procedure is the trapping of particles in ghosts of human erythrocytes. This is achieved by the gradual hemolysis of erythrocytes in the presence of the particles to be trapped. The second step is the Sendai virus-induced fusion of the ghosts containing the particles with cells. By this method, ferritin and latex spheres (diameter 0.1 μm) have been “injected” into cells.

The ultimate aim of a great deal of biological research is to understand the function of various types of macromolecules within the intact cell. Since many aspects of the intracellular behavior of macromolecules cannot adequately be studied in cell extracts, many laboratories have tried with varying degrees of success to introduce isolated macromolecules into mammalian cells in order to examine their behavior (1–8). A drawback to many of these experiments is that it is not always simple to introduce large molecules into cells efficiently, or to prevent the molecules' breakdown during entry (1, 2, 6, 7). Macromolecules have been introduced into cells by spontaneous uptake, assisted in the case of nucleic acids and proteins by polycations (6, 7, 9). Liposomes containing trapped enzyme have been used as a vehicle for introducing enzyme molecules into mammalian cells (10). When macromolecules were taken up by cells spontaneously or with the aid of liposomes, it was not clear whether they entered directly into the cytoplasm or indirectly by means of endocytosis (2, 7, 10). The most efficient technique currently

used is microinjection which has the advantage of introducing material directly into the cytoplasm, but has the disadvantage of being laborious, particularly when applied to normal-sized cells (3–5, 8).

It seemed likely that a method for the simultaneous “injection” of large numbers of cells could easily be developed on the basis of previous findings on virus-induced fusion. Investigations in this laboratory showed that, under suitable conditions using Sendai virus, erythrocytes could be fused with other cells without loss of erythrocyte contents (11). In these experiments the cytoplasm of the erythrocyte mixed rapidly with the cytoplasm of the recipient cell. Similar observations have been recently reported by Furusawa et al. (12). Since trapping of macromolecules and small particles in human erythrocyte ghosts had been demonstrated previously (13, 14), it seemed of interest to try to fuse ghosts containing trapped particles with other cells. Trapping of particles within ghosts followed by fusion of the ghosts with cells would constitute a means of “injecting” particles into the cytoplasm of the cell. This aim

has been achieved in the experiments described below. An abstract of the present work has appeared elsewhere (15).

MATERIALS AND METHODS

Salt Solutions

The medium used for the suspension of human erythrocytes contained 160 mM NaCl and 20 mM Tricine-NaOH buffer, pH 7.4 (solution Na).¹ The hypotonic medium used for dialysis of the erythrocytes contained 40 mM NaCl and 10 mM Tricine-NaOH buffer, pH 7.4. The medium used for fusion experiments contained 128 mM KCl, 32 mM NaCl, and 20 mM Tricine-NaOH buffer, pH 7.4 (solution K).

Cells

Human blood, type O, aged 3–6 wk was used. The blood cells were washed three times with solution Na, the buffy layer containing white cells was discarded and the pellet was suspended in solution Na to give a concentration of 10% (vol/vol).

Hepatoma tissue culture (HTC) cells, subclone GM 22-5, were grown as described previously (16). The cells were washed twice in solution K and finally suspended in this solution to give a concentration of 10% (vol/vol).

Virus

Sendai virus was isolated and its hemagglutinin titer was determined as described previously (17).

Conjugation between Fluorescein

Isothiocyanate and Bovine Serum

Albumin (BSA)

Fluorescein isothiocyanate (2.5 mg), dissolved in 0.13 ml of 0.5 M carbonate-bicarbonate buffer pH 9.7, was added to 100 mg of BSA dissolved in 1 ml of water. The solution was stirred for 3 h at room temperature and the free fluorescein isothiocyanate was removed by dialysis overnight in the cold. Fluorescence intensity was measured on an Eppendorf Fluorometer, using a 265–366-nm filter for excitation and a 530–3,000-nm filter for emission.

Trapping of BSA in Ghosts

Erythrocytes, 2.5 ml of a 10% (vol/vol) suspension, were mixed with 7.5 ml of solution Na containing 150 mg BSA and the resultant suspension was dialyzed for 2 h

against 1 or 2 liters of hypotonic medium. At the end of the dialysis, 0.5 ml of a solution containing 2.2 M NaCl and 0.02 M MgSO₄ was added, and the suspension was incubated with gentle shaking for 30 min at 37°C. After incubation, intact cells were removed by centrifugation at 260 g for 7 min, and the ghosts which remained in the supernate were collected by centrifuging at 17,300 g for 15 min. The pellet obtained was washed twice with solution Na and finally suspended in 1 ml of either solution Na or solution K.

Fusion of Ghosts with HTC Cells

HTC cells, 0.2 ml of a 10% (vol/vol) suspension, and 0.2 ml of ghost suspension, prepared as described above, were mixed in 25-ml scintillation vials in a final volume of 1 ml of solution K which contained 1 mM MnCl₂. Sendai virus (1,600 hemagglutinating units [HAU]) was then added, and the cell suspension was incubated in the cold for 10 min to allow agglutination. To obtain fusion, the vials were incubated with gentle shaking at 37°C for 10–20 min, and the process was terminated by cooling.

Preparation of Samples for

Electron Microscopy

Samples were prepared for electron microscopy as described previously (18), except that, in experiments where latex particles were present, the samples were embedded in Spurr epoxy resin (19). Sections of preparations containing ferritin were stained only with uranyl acetate and not with lead citrate.

Materials

Polystyrene latex 0.109 μm in diameter was obtained from the Dow Chemical Co., Midland, Mich.; ferritin 2 \times crystallized, trace cadmium (horse spleen) was obtained from Pentex Biochemical, Kankakee, Ill. Albumin (bovine) fraction V, B grade was obtained from Calbiochem, San Diego, Calif.; fluorescein isothiocyanate isomer I was obtained from Sigma Chemical Co., St. Louis, Mo. T₂ phage was kindly donated by Prof. U. Bachrach and Dr. R. Levin of the Bacteriology Department of the Hebrew University-Hadassah Medical School.

RESULTS

Trapping of Ferritin, Bacteriophage, and Latex Spheres in Ghosts

Particles added to the external medium of human erythrocytes during gradual hemolysis in the presence of BSA were trapped within the resultant ghosts. Particles shown to be trapped in this way within ghosts included ferritin (diameter = 120 Å, Fig. 1 *a*), bacteriophage T₂ (diameter of head = 650 Å, Fig. 1 *b*), and latex spheres (diam-

¹ *Abbreviations used in this paper:* BSA, bovine serum albumin; HAU, hemagglutinating units; HTC, hepatoma tissue culture; solution K, 128 mM KCl, 32 mM NaCl, and 20 mM Tricine-NaOH buffer, pH 7.4; solution Na, 160 mM NaCl and 20 mM Tricine-NaOH buffer, pH 7.4.

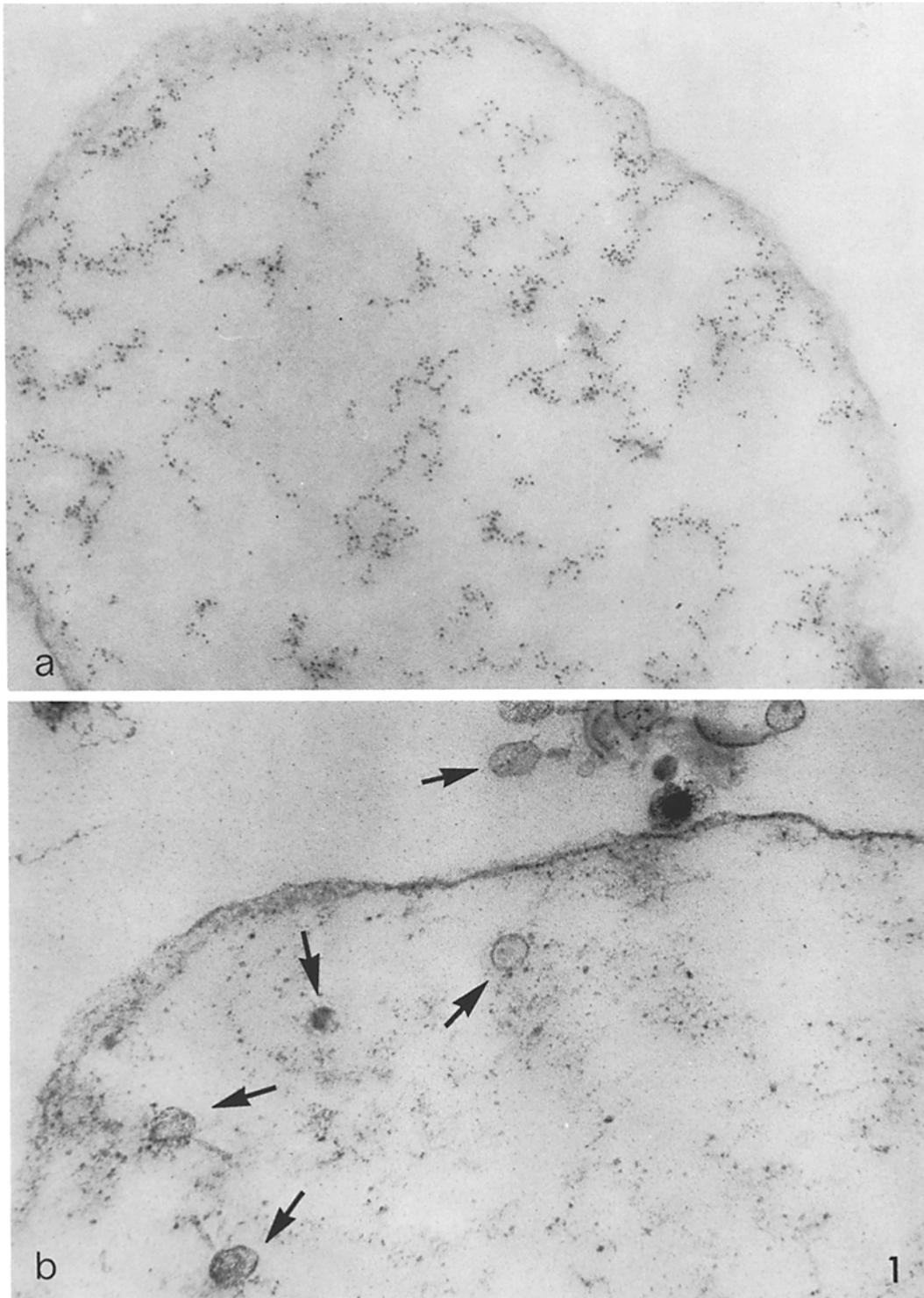


FIGURE 1 *a* and *b* Trapping of ferritin and T₂ phage in human erythrocyte ghosts. (*a*) Trapping of ferritin. Erythrocytes, 0.5 ml of a 50% (vol/vol) suspension, were mixed with 2 ml of solution Na containing 37.5 mg of BSA and 100 mg of ferritin. All other steps were as described for trapping of BSA in Materials and Methods. $\times 84,150$. (*b*) Trapping of T₂ phage. Erythrocytes, 0.5 ml of a 20% (vol/vol) suspension, were mixed with 1 ml of solution Na containing 30 mg of BSA and 0.5 ml of T₂ suspension containing 10^{12} phage particles/ml. All other steps of dialysis and resealing of ghosts were as described in Materials and Methods. Phage particles can be seen outside and inside the ghosts (arrows). $\times 64,750$.

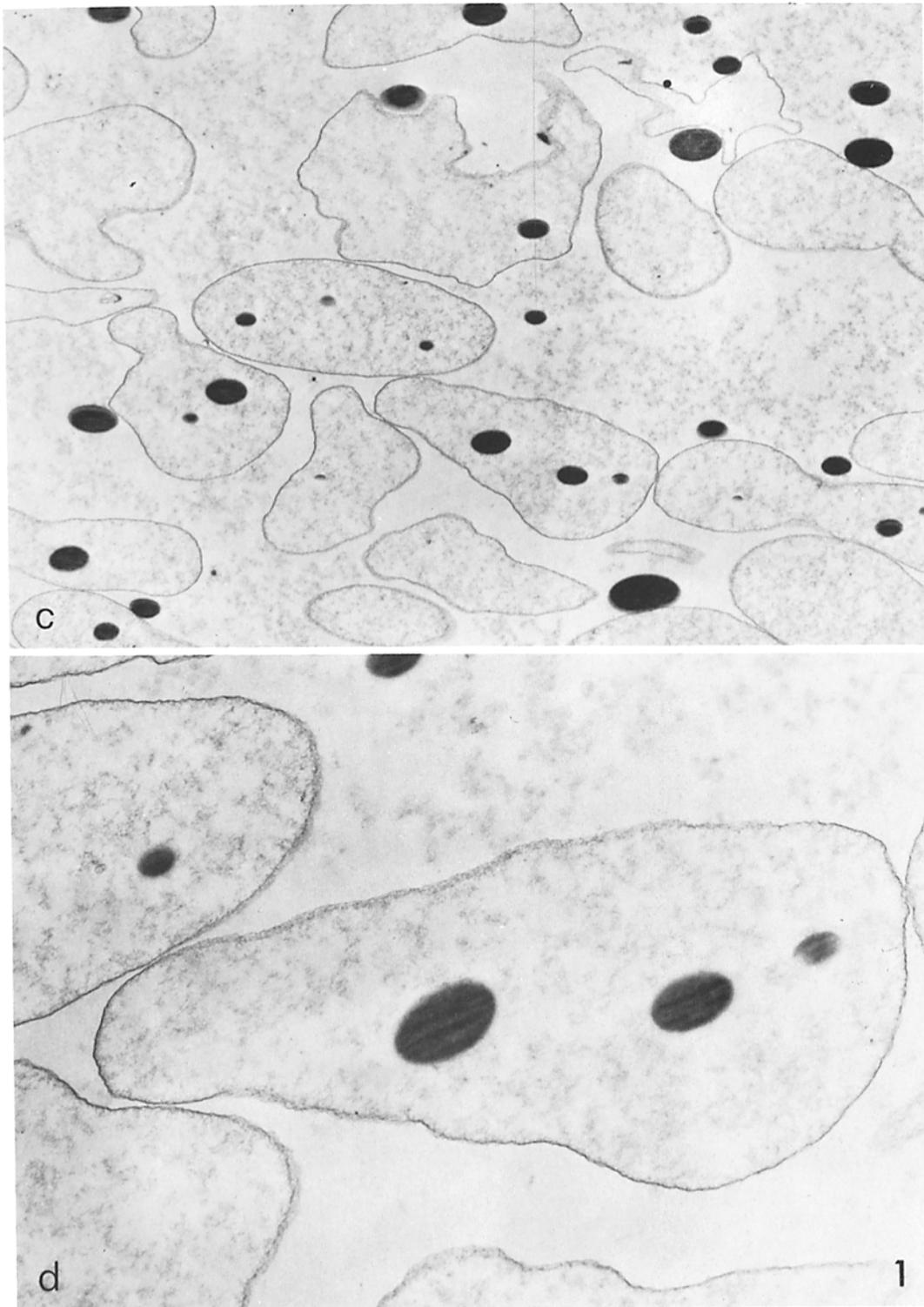


FIGURE 1 *c* and *d* Trapping of latex particles in human erythrocyte ghosts. (*c*) Trapping of latex. All steps of trapping and dialysis were as described for "Trapping of BSA" (see Materials and Methods), except that 1 ml (100 mg) of latex particles of $0.109\ \mu\text{m}$ was added to the cell suspension before dialysis. A crowded field of resealed ghosts containing latex. $\times 8,575$. (*d*) High magnification picture of a ghost containing latex particles. $\times 24,500$.

eter = 0.1 μm , Fig. 1 *c, d*). Although the size of the latex spheres specified by the manufacturer was 0.1 μm , the diameter of the latex particles seen in the electron micrographs was as high as 0.7 μm . Measurement of the diameter of latex spheres in the initial suspension by negative staining confirmed that their diameter was 0.1 μm . The reason for the discrepancy between the diameter of latex spheres in the original suspension and that seen in the embedded material is not clear. The most likely explanation seems to be that the latex spheres changed in size during fixation and embedding of the ghosts. These findings indicate that holes of at least 0.1 μm are formed in erythrocytes during gradual hemolysis in the presence of BSA.

Ghosts which had trapped latex particles were transferred to isotonic medium and incubated with ferritin. In most of the ghosts containing latex, no ferritin was observed. This indicates that holes in most of the ghosts were resealed under the conditions used (Fig. 2).

Virus-Induced Fusion of Ghosts and HTC Cells

Ghosts could be fused with HTC cells with considerable efficiency. Leakage of ghost contents during fusion was measured by using ghosts containing fluorescein-labeled BSA (Table I). Since Mn^{++} was previously found to reduce virus-induced hemolysis during fusion of intact human

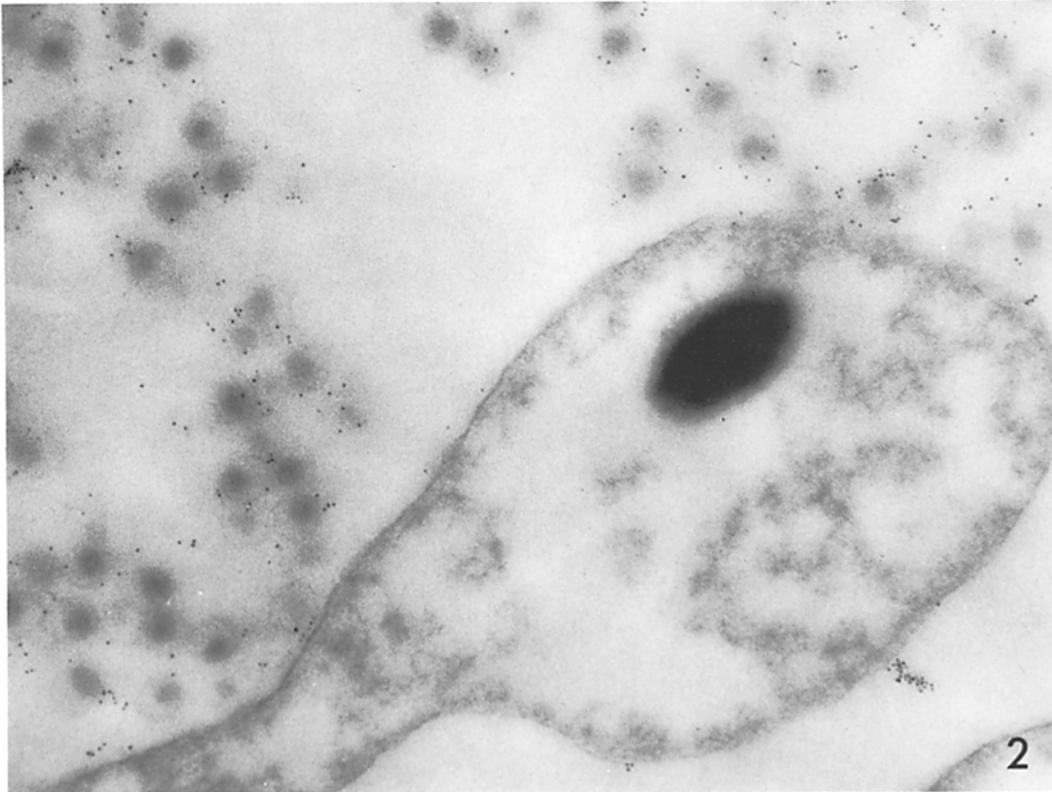


FIGURE 2 Exclusion of ferritin by ghosts containing latex particles. Latex particles were trapped in ghosts as described in the legend to Fig. 1. The final pellet of the ghosts in which latex particles were trapped was suspended in solution Na, containing 30 mg/ml of ferritin, to give a concentration equivalent to 1.5% (vol/vol) of original erythrocytes. The ghosts were incubated in the presence of ferritin with gentle shaking for 20 min at 37°C. At the end of the incubation period, 1 ml of the suspension was fixed for 2 h at 0°C with 1 ml of 4% glutaraldehyde dissolved in 160 mM NaCl and buffered with 20 mM of sodium cacodylate, pH 7.4. All other steps were as described in preparation of samples for electron microscopy. Note ferritin particles scattered only outside the erythrocyte ghost.

TABLE I
Leakage of Fluorescein-Labeled BSA from Ghosts during Fusion of the Ghosts with HTC Cells

Addition	HTC cells fused with ghosts*	
	%	Hemolysis %
None	0	2
Virus	10-20	27
Virus + MnCl ₂	20-40	22

Ghosts containing fluorescein-labeled BSA were prepared as follows. Erythrocytes, 5 ml of a 20% (vol/vol) suspension, were suspended in 15 ml of solution Na containing 225 mg of fluorescein isothiocyanate BSA and 75 mg BSA. All other steps of dialysis and resealing of ghosts were as described under Materials and Methods. Each fusion system contained 0.2 ml of HTC cells 10% (vol/vol) suspension and 0.2 ml of ghosts filled with fluorescein-labeled BSA at a concentration equivalent to 25% (vol/vol) of original erythrocytes suspended in a final volume of 1 ml of solution K containing 1 mM MnCl₂. The virus concentration was 1,600 HAU/ml. All other details are described under Materials and Methods. * It was impossible to measure the exact fusion index in the systems and therefore a rough estimate of the extent of fusion based on microscope observation is given.

erythrocytes with other cells, its effect was tested here. Table I shows the percent fusion of ghosts with HTC cells and the leakage of fluorescein-labeled BSA after incubation with Sendai virus in the presence and absence of Mn⁺⁺. Ghosts fused with HTC cells in the absence of added bivalent cation, but Mn⁺⁺ considerably increased the percent of fusion.

Fig. 3 shows a phase micrograph of ghosts fused with HTC cells. Since fusion was performed in the presence of an excess of ghosts, very few homopolykaryons were observed. The protrusions (G) on the cells which had fused with ghosts usually retained the outline of the erythrocyte membrane. The opacity of these protrusions suggested that the HTC cell contents had flowed into the ghost cavity.

Introduction of Ferritin and Latex Particles into HTC Cells by Fusion

When ghosts containing latex particles or ferritin were fused with HTC cells, the particles trapped within the ghosts could be detected in the cytoplasm of the fused cells. Fig. 4 *a* shows a latex

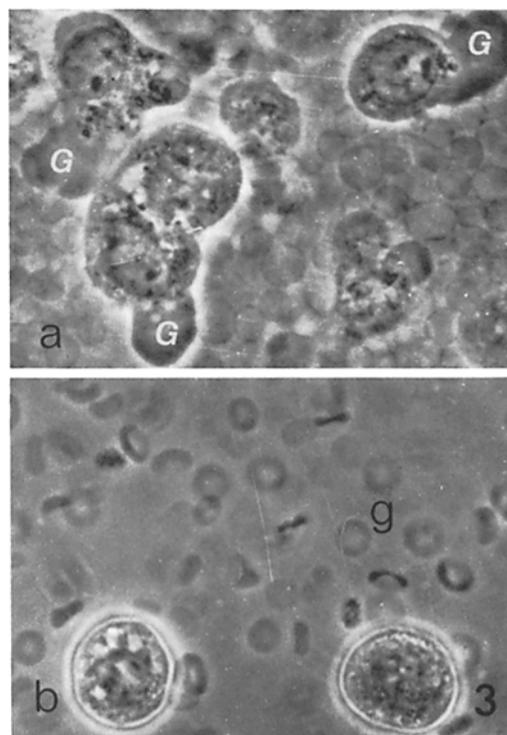


FIGURE 3 Fusion of HTC cells with human erythrocyte ghosts (a) Fusion was performed as described in Materials and Methods in the presence of 1,600 HAU/ml of virus and 1 mM MnCl₂. After 15 min at 37°C, cells were photographed. Ghosts and cells are in tight agglutination. Some of the ghosts (G) have fused with HTC cells. × 120. (b) A suspension of HTC cells and human erythrocyte ghosts in the absence of added virus (control). Cells were photographed after 15-min incubation at 37°C. g, ghosts. × 120.

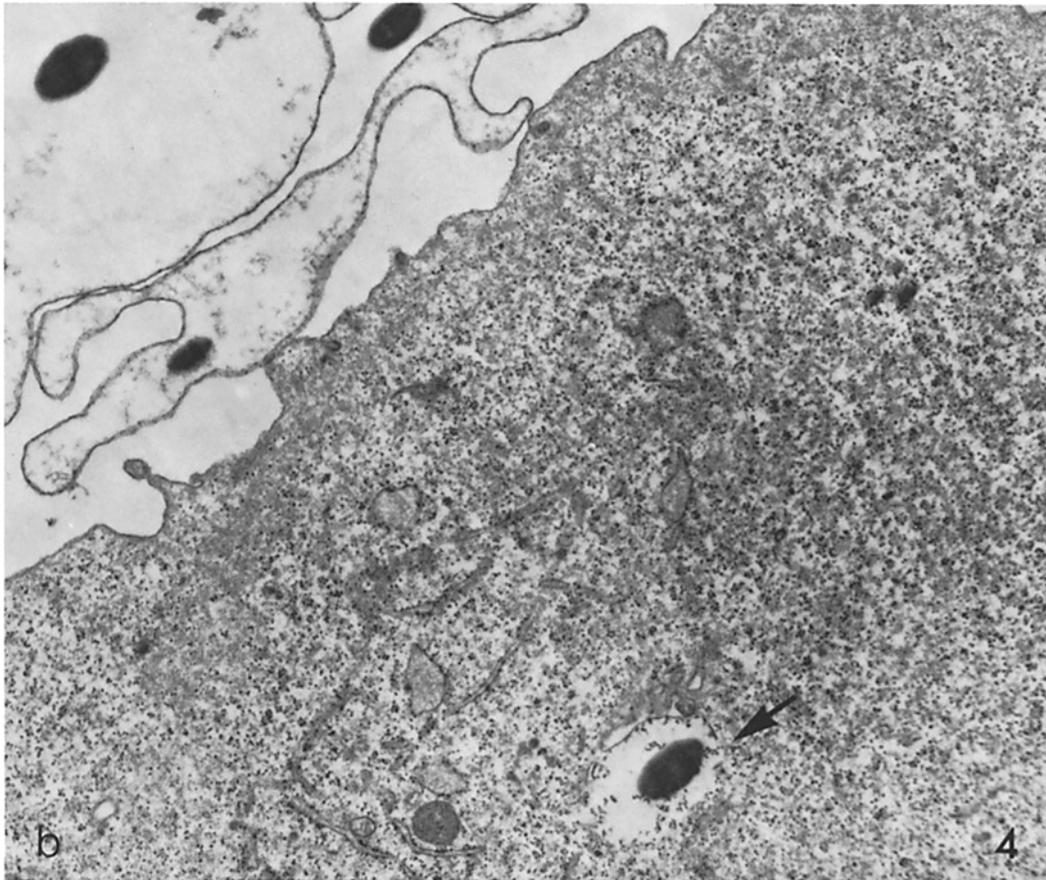
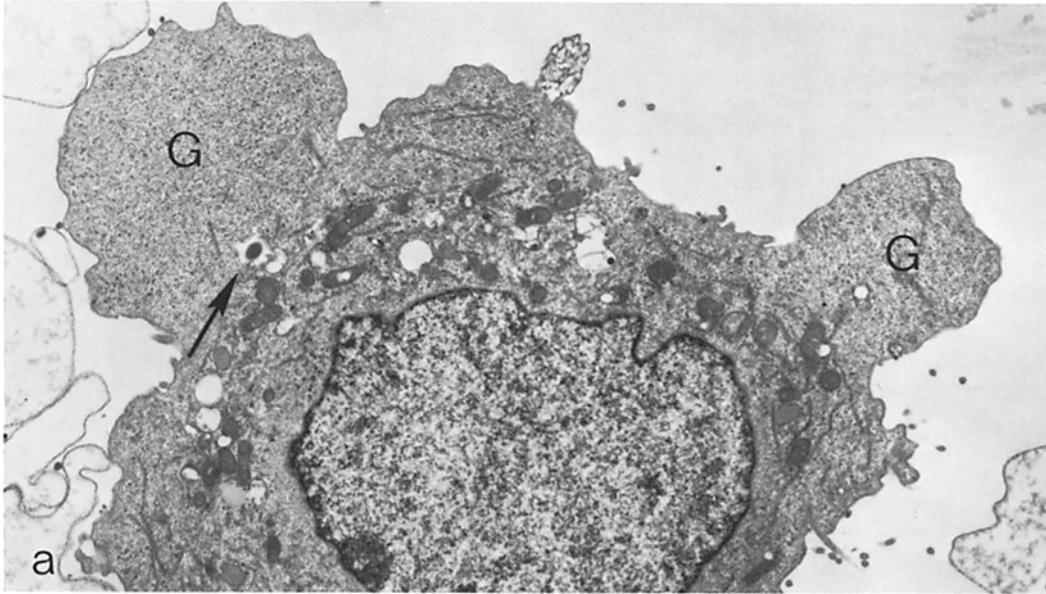
particle within the cytoplasm of an HTC cell which had fused with ghosts containing latex. The protrusions (G) on the cell (Fig. 4 *a*), which are presumably ghosts fused to the cell, are full of cytoplasm, including ribosomes, but interestingly contain no mitochondria. Fig. 4 *b* shows a high magnification of another cell containing a latex particle. In certain cases the latex particle was surrounded by a clear region as in Fig. 4 *b*. It should be noted that numerous polyribosomes are in contact with the latex. Adjacent to the latex particles there is a fragment of membrane, presumably originating from the endoplasmic reticulum since it has polyribosomes attached to it. In this figure a ghost containing latex is seen adjacent to the cell.

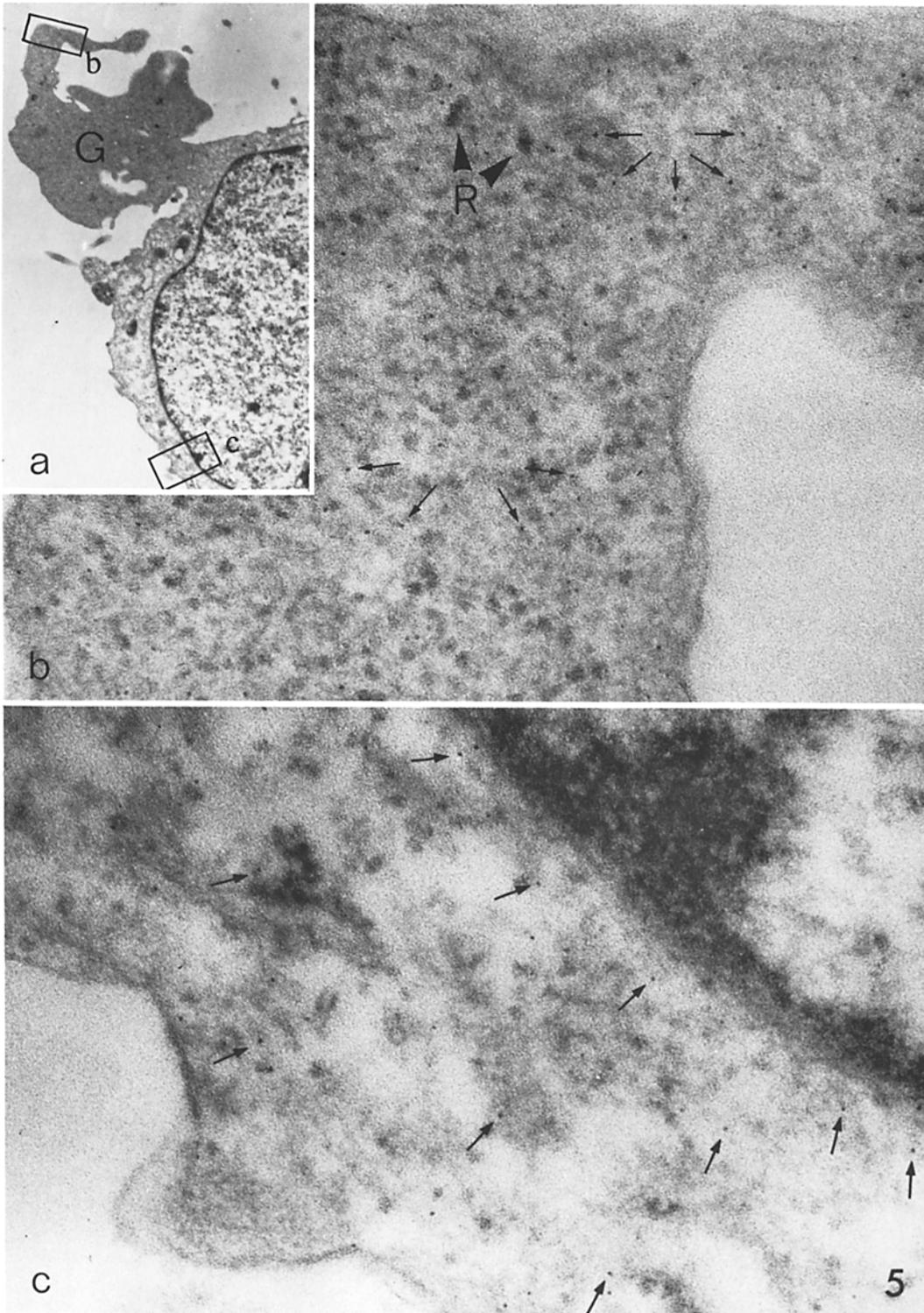
FIGURES 4-6

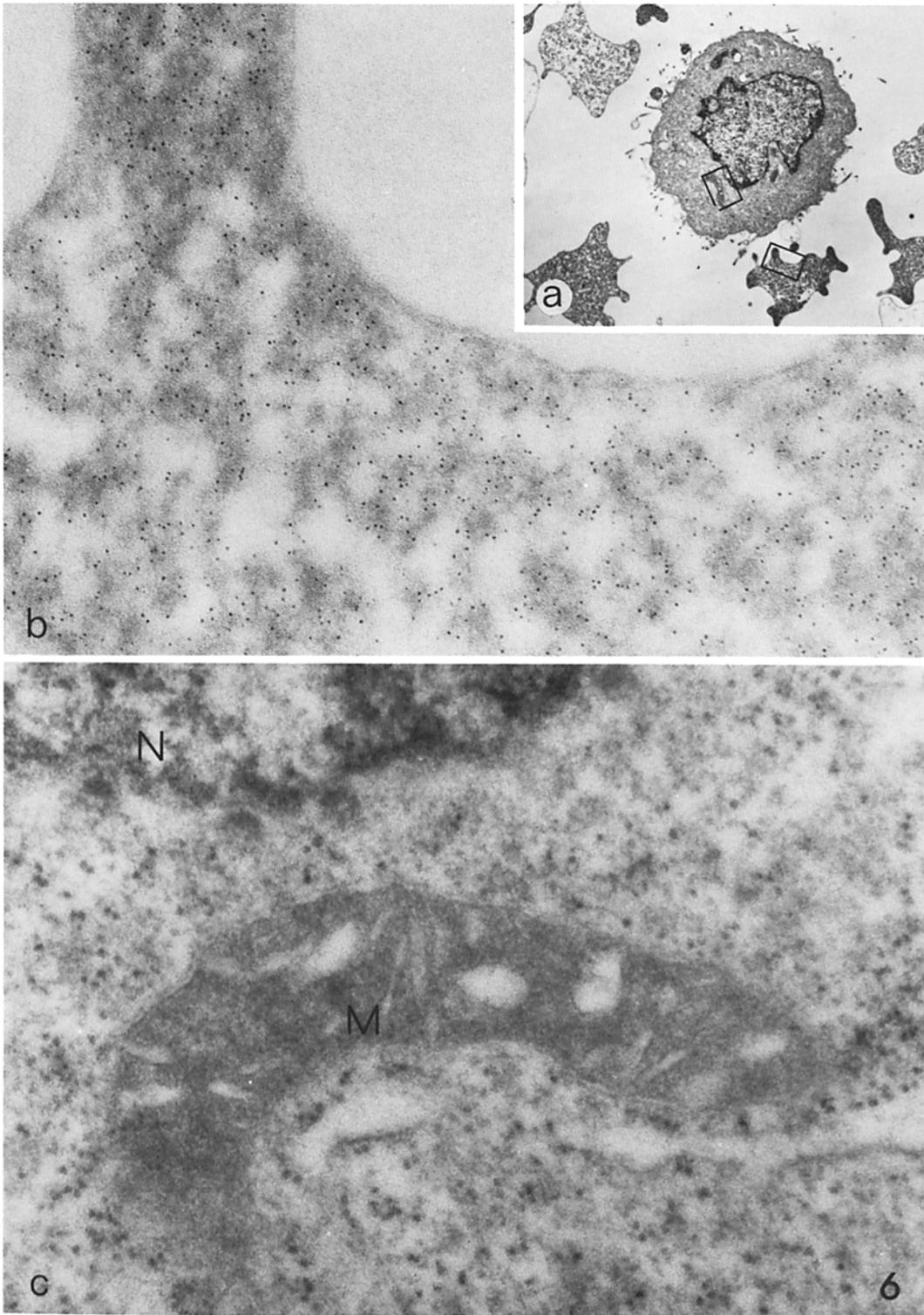
FIGURE 4 Introduction of latex particles into HTC cells. All experimental conditions were as described under **Materials and Methods** and in the legend to Fig. 1 c. (a) An HTC cell after fusion with a preparation of erythrocyte ghosts in which latex particles had been trapped. The two projections (*G*) on the HTC cell are presumably fused ghosts. A latex particle (arrow) can be seen within the fused cell. Note that the lumen of the former erythrocyte ghost is full of ribosomes but without any mitochondria or endoplasmic reticulum. $\times 7,585$. (b) High magnification of part of an HTC cell containing a latex particle (arrow). Note that some ribosomes are attached to the latex particles. A ghost containing a latex particle can be seen in contact with the HTC cell. $\times 16,830$.

FIGURE 5 Introduction of ferritin particles into HTC cells. Ferritin was trapped in ghosts by suspending 2.5 ml of 10% (vol/vol) erythrocytes in 7.5 ml of solution Na containing 150 mg BSA and 100 mg ferritin followed by dialysis against hypotonic medium. Details of dialysis, resealing and fusion are described under **Materials and Methods**. The preparation of samples for electron microscopy was as described in **Materials and Methods**, except that the samples were stained in uranyl acetate in Michaelis buffer immediately after fixation as described previously (23), and the sections were again stained with uranyl acetate. (a.) A low magnification picture showing an HTC cell fused with a ghost (*G*). Note that contents of the ghost lumen are denser than the contents of the main body of the cell. $\times 5,325$. (b) Enlargement of the area marked *b* in Fig. 5 a. Many ribosomes (*R*) and ferritin particles (arrows) can be seen within the lumen surrounded by the ghost membrane. $\times 87,450$ (c) Enlargement of the area marked *c* in Fig. 5 a. Note that ferritin particles (arrows) can be seen in close proximity to the nuclear membrane but none are seen inside the nucleus. $\times 87,450$.

FIGURE 6 HTC cells incubated with ghosts containing ferritin, in the absence of Sendai virus. Ferritin was trapped in ghosts by suspending 0.5 ml of erythrocytes 50% (vol/vol) in 2.5 ml of solution Na⁺ containing 37.5 mg BSA and 100 mg ferritin, followed by dialysis against hypotonic medium. Details of dialysis, resealing of ghosts, and preparation of samples for electron microscopy were as described in **Materials and Methods**. The cells were incubated at 4°C and 37°C in the absence of Sendai virus. (a) A low magnification picture showing an HTC cell surrounded by ghosts. $\times 3,840$. (b) Enlargement of the area of the ghost marked in Fig. 6 a. Many ferritin particles may be seen enclosed by the ghost membrane. $\times 78,750$. (c) Enlargement of the marked area of the HTC cells in Fig. 6 a. No ferritin particles can be seen in the HTC cell. *N*, nucleus. *M*, mitochondria. $\times 78,750$.







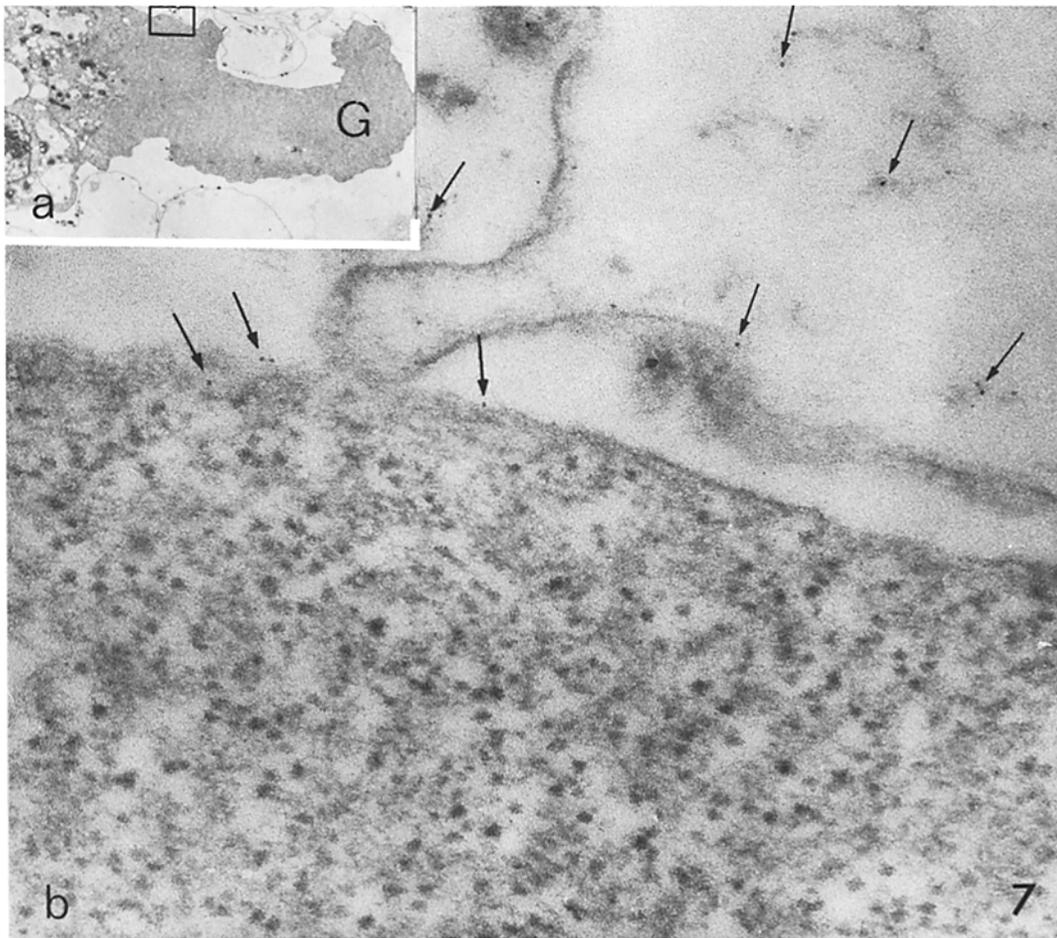


FIGURE 7 Exclusion of ferritin by HTC cells fused with ghosts containing BSA in the presence of external ferritin. BSA was trapped in ghosts by incubating 0.5 ml of erythrocytes (50% [vol/vol]) in 2.5 ml of solution Na^+ containing 37.5 mg BSA, followed by dialysis against hypotonic medium. Details of dialysis, re-sealing of ghosts, fusion and preparation of samples for electron microscopy were as described in Materials and Methods. Cells were fused in the presence of 1 mg/ml of ferritin in the suspending medium. (a) A low magnification picture of an HTC cell fused with a ghost (G). $\times 2,640$. (b) Enlargement of the area of the HTC cell marked in Fig. 7 a. A few ferritin particles (arrows) can be seen outside the HTC cell and inside the ghost. No ferritin particles are seen in the HTC cell. $\times 87,450$.

When ghosts containing ferritin were fused with HTC cells, large numbers of ferritin particles were found in the cytoplasm of the fused cell (Fig. 5). Fig. 5 a shows a low magnification electron micrograph of a ferritin-filled ghost (G) fused with an HTC cell. The outline of the fused ghost is still clearly visible. In this cell and in many others examined, the cytoplasm in the interior of the former ghost (G) is much more electron dense than the cytoplasm of the fused HTC cell. This might

indicate that there has been a strong flow of HTC cell cytoplasm into the lumen of the ghost, as indicated by the presence of large numbers of ribosomes (Fig. 5 b). Fig. 5 b shows a high magnification of a part of the cell in which ferritin particles are clearly seen alongside ribosomes. Fig. 5 c shows ferritin particles in a portion of the cytoplasm adjacent to the nucleus and distant from the point of fusion. It should be noted that no ferritin particles were seen in the nucleus. Most, if

not all, of the cells in the preparation which had fused with ghosts contained ferritin. Fig. 6 shows a control experiment in which ferritin-filled ghosts were incubated with HTC cells in the absence of virus. While the ghosts remained full of ferritin (Fig. 6 *b*) no ferritin could be detected in the HTC cells (Fig. 6 *c*). In another control experiment (Fig. 7) ghosts containing only BSA were fused with HTC cells, and ferritin was added to the external medium during fusion. No ferritin particles were detected in the cytoplasm of cells fused with ghosts in these experiments, although a few ferritin particles entered some of the ghosts because of virus-induced lysis (Fig. 7 *b*).

DISCUSSION

This paper confirms the observations of other laboratories that soluble macromolecules and particles can become enclosed in ghosts during lysis of human erythrocytes (13, 14). Our experiments, which show entry of latex particles and bacteriophages into ghosts, indicate that holes as large as 0.1 μm are produced in the membrane during gradual hemolysis. Seeman (14) previously showed the entry of colloidal gold particles of up to 300 \AA in diameter into ghosts but did not report attempts to introduce larger particles. Baker, on the other hand, was unable to detect any gold particles within ghosts formed by the hemolysis of erythrocytes in the presence of colloidal gold particles of 250 \AA diameter (20). It is possible that the conditions of hemolysis are crucial in determining the size of the holes formed in the erythrocyte membrane as well as their rate of resealing (see, for example, reference 21).

The main finding of the present investigation is that particles trapped within erythrocyte ghosts can be introduced into other cells by virus-induced fusion. We suggest the name "ultramicroinjection" for the technique. The rapid entry of cell cytoplasm into the lumen of the ghost after fusion shows that there is no barrier between the ghost and cell compartments. Thus, our method leads to the direct entry of foreign particles or macromolecules into the cytoplasm of the recipient cells. While this manuscript was in preparation, Furusawa et al. reported the "injection" of a small molecule, fluorescein isothiocyanate, into mammalian cells by a similar technique (12). The method proposed here achieves the same goal as direct microinjection and permits large numbers of cells to be "injected" with ease. Some dis-

advantages of our method are that it is wasteful of the material injected, that it results in the introduction of material other than that to be tested into the cell, such as residual hemoglobin or trapped BSA, and that it requires Sendai virus which even when inactivated might have undesirable effects on cells.

The possible applications of the method are numerous and include introduction of DNA, RNA, and enzymes into cells to alleviate genetic defects (2). The method may also be useful for introducing fragments of chromosomes into cells (22). Another possible application of the technique might be the introduction of viruses into cells not normally susceptible to them.

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