

Intracellular Colocalization of Variant Surface Glycoprotein and Transferrin–Gold in *Trypanosoma brucei*

Paul Webster and Dennis J. Grab

International Laboratory for Research on Animal Diseases, Nairobi, Kenya

Abstract. Endocytosis and intracellular transport has been studied in the bloodstream forms of *Trypanosoma brucei* by light and electron microscopy, using colloidal gold coupled to bovine transferrin (transferrin–gold). The endocytosed transferrin–gold, visualized by silver intensification for light microscopy, was present in vesicular structures between the cell nucleus and flagellar pocket of the organism. At the ultrastructural level, transferrin–gold was present after a 10-min incubation in the flagellar pocket, coated vesicles, cisternal networks, and lysosomelike structures. Endocytosis and intracellular processing of *T. brucei* variable surface glycoprotein (VSG) was studied using two preparations of affinity-purified rabbit IgG directed against different parts of the VSG. One preparation of IgG was directed against the cross-reacting determinant (CRD): a complex glycolipid side chain covalently linked to the COOH-terminus of the VSG molecule. The other was directed against determinants on the

rest of the VSG molecule. When the two IgG preparations were used on thawed, thin cryosections of trypanosomes that had been incubated in transferrin–gold before fixation, the organelles involved with transferrin–gold endocytosis labeled with both antibodies, as well as many vesicular, tubular, and vacuolar structures that did not contain endocytosed transferrin–gold. Both antibodies also labeled the cell surface. In double-labeling experiments both antibodies were closely associated except that IgG directed against the VSG molecule labeled all the cisternae of the Golgi apparatus, whereas anti–CRD IgG was shown to label only half of the Golgi apparatus. Evidence for sorting of VSG molecules from endocytosed transferrin–gold was found. Double-labeling experiments also showed some tubular profiles which labeled on one side with anti–CRD IgG and on the other side with anti–VSG IgG, suggesting a possible segregation of parts of the VSG molecule.

A protozoan, parasitic blood flagellate of livestock and wildlife in Africa, *Trypanosoma brucei*, develops in and is transmitted by tsetse flies (*Glossina* spp.). The success of trypanosomes in avoiding the immune response of their hosts lie in their ability to vary, both biochemically and antigenically, the glycoprotein coat (variable surface glycoprotein [VSG])¹ covering the entire cell surface during the course of infection in the mammalian host (8, 9, 31).

Trypanosome VSGs can be isolated in two distinct forms: an amphiphilic membrane form (mfVSG) isolated by solubilization of trypanosomes in boiling detergent (6, 7) or Zwittergent (15, 18, 19), and a soluble form (sVSG) recovered after mechanical or osmotic disruption of the cells (8). The membrane form contains glycosyl-*sn*-1-2-dimyristyl phosphatidylinositol at its COOH-terminus (11) which is lacking in sVSG. The conversion of mfVSG to sVSG (6) is believed

to be effected by an endogenous, phospholipase C-like enzyme (mfVSG phospholipase) which cleaves dimyristyl glycerol from the terminal phosphatidylinositol (11, 23). It has been postulated that this enzyme is responsible for VSG release from the plasma membrane when VSG-coated bloodstream forms transform, in the insect vector, to forms lacking VSG (4, 7, 10, 11).

The site of the immunological cross-reactivity reported between sVSGs of different species of African trypanosomes (*T. brucei* and *T. congolense*) resides in a complex glycolipid side chain located near the COOH-terminus of the protein (2, 21). This constitutes the cross-reacting determinant or CRD. Antibodies to the CRD are claimed to recognize their ligand only after the VSGs have been converted to sVSG (6, 10). However, unpublished results (Turner, M. J., quoted in reference 23) suggest that CRD antibody has a higher affinity for sVSG but will still bind mfVSG. Antibodies to the CRD have been used to demonstrate a *trans*-Golgi localization for the addition of CRD to VSG (15). However, the latter study conflicts with other published data suggesting that the CRD is added to the VSG molecule in the endoplasmic reticulum (1, 11).

It is believed that the surface glycoproteins of trypano-

Address correspondence to Paul Webster, ILRAD, P.O. Box 30709, Nairobi, Kenya.

1. *Abbreviations used in this paper:* CRD, cross-reacting determinant; mfVSG, membrane form variable surface glycoprotein; PAG, protein A–colloidal gold; sVSG, soluble form variable surface glycoprotein; VSG, variant surface glycoprotein.

somes are too antigenically diverse to be useful as a material for a vaccine (27). However, other antigenically sensitive sites vulnerable to antibody attack may be revealed as the cell biology of the organism is studied. A process which may serve as a target for antibody attack within trypanosomes is endocytosis. In trypanosomes the process of endocytosis seems to occur only from the specialized membrane of the flagellar pocket, a deep invagination of the cell plasma membrane where the flagellum leaves the body of the cell (25). It is a closed pocket that appears to be cut off from the surrounding environment by a close apposition of plasma membrane and flagellar membrane. It is clear, however, that various particulate and soluble substances in the extracellular medium can enter the flagellar pocket (3, 25). The electron-dense marker, ferritin, has been shown to enter the flagellar pocket and be taken up by the cell in cytoplasmic coated vesicles that bud from the flagellar pocket membrane (25). These vesicles were shown to fuse with and discharge their contents into a tubular membrane system that connected with multivesicular bodies and structures believed to be digestive vacuoles (25). The vesicles and tubular networks were shown to contain an electron opaque substance similar in appearance to the VSG (or surface coat). However, no VSG-like material was reported in the digestive vacuoles (or lysosomelike structures).

These endocytotic processes are comparable with endocytotic processes in other cells (27, 28) where clathrin-coated vesicles originating from the cell surface carry endocytosed markers to intermediate organelles, the endosomes (20), which then discharge their contents into lysosomes. In mammalian cells, endosomes are prelysosomal compartments responsible for much of the molecular sorting in the endocytosis pathway.

In trypanosomes the term 'endosome' was originally used to refer to the nucleolus of the nucleus but this usage seems to have been dropped in recent years. In this report the term endosome is used in the more modern sense of an organelle involved in the uptake and processing of extracellular material. The lack of suitable immunochemical or cytochemical markers has made the identification of lysosomal and hence prelysosomal compartments difficult in trypanosomes.

Ultrastructural studies of transferrin uptake in mammalian cells have demonstrated rapid recycling of the protein and its receptor back to the cell surface after receptor-mediated endocytosis (24). The normal intracellular pathway of transferrin is altered, however, if it is coupled to colloidal gold particles. Instead of recycling, the marker is delivered to lysosomes (26, 32). As transferrin uptake by trypanosomes appears not to have been studied before, we have used transferrin-colloidal gold complexes (transferrin-gold) to identify parts of the endocytosis pathway in *T. brucei*. We have also used antibodies to study the intracellular localizations of VSG and CRD. A colocalization of VSG and endocytosed transferrin-gold has been demonstrated using the rabbit IgG preparations to the VSG molecule. In addition, evidence is presented indicating segregation of VSG from endocytosed transferrin-gold and the possibility of intracellular VSG processing is demonstrated. The structures involved with this sorting are postulated as being prelysosomal, probably part of an endosome compartment.

Materials and Methods

Materials

Glutaraldehyde was supplied by Agar Aids Ltd., Essex, England; RPMI 1640 from Flow Laboratories, Irvine, Scotland; staphylococcal protein A from Pharmacia Inc., Uppsala, Sweden; Pipes and gelatin (from calf skin) from Sigma Chemical Co., London, England. FBS was from HyClone Laboratories, Logan, UT. ^{125}I -labeled protein A (1.1 GBq/mg) was from Amersham International, Amersham, England. Dulbecco's PBS was from Gibco, Paisley, Scotland. RX medical x-ray film was from Fuji Photo Film Co., Japan. Spectraphor-2 (12,000–14,000 mol wt cut-off) dialysis membrane tubing was from Spectrum Medical Industries, Inc., Los Angeles, CA. IntenSe silver intensification kit was from Janssen Pharmaceutica, Beerse, Belgium. All other chemicals were supplied by BDH Chemicals, Poole, England.

Parasites

Trypanosomes were grown from cryopreserved stabilates in irradiated (600–900 rads) rats and isolated from infected blood by isopycnic density centrifugation on Percoll gradients (13). Immunolabeling experiments were carried out on cryosections of *T. brucei* (MITat 1.2) populations. Control experiments were performed on frozen sections of *T. brucei* (ETat 1.3), *T. brucei* (MITat 1.52) and *T. congolense* (ILNat 2.1) were used for immunoblot analysis of IgG preparations.

Antibodies

The preparation and characterization of the affinity-purified rabbit polyclonal IgG preparations to the VSG of MITat 1.2 (RxVSG) and to the cross-reacting determinant (RxCRD) have been described (15).

Immunoblot Analysis

Immunoblot analysis was conducted as described by Burnette (5). In brief, SDS-PAGE was performed with either protein from whole trypanosomes (30 μg protein per slot) or with soluble form VSG (5 μg protein per slot) prepared as described by Cross (8). The proteins were electrophoretically transferred on to a nitrocellulose filter and the filter, after blocking with excess BSA, was incubated overnight at 4°C with $\sim 3 \mu\text{g}$ affinity-purified IgG/10 ml of PBS. Antibody bound to the nitrocellulose was detected using ^{125}I -labeled protein A and autoradiographed as described (5).

Preparation of Colloidal Gold Probes

Monodisperse colloidal gold sols of four homogeneous sizes (5, 8, 10, and 15 nm) were produced using the method of Slot & Geuze (30). The 5-nm colloidal gold sol was coupled to 10 μg of bovine transferrin per 1 ml of gold sol at pH 5 using established methods (22). Before coupling to gold, bovine transferrin was dissolved at a concentration of 40 $\mu\text{g}/\text{ml}$ in 50 mM sodium phosphate buffer (pH 7) and dialyzed against the same buffer to remove any low-molecular weight impurities. All other gold sols were coupled to staphylococcal protein A using the same methods. Further purification of all colloidal gold probes was carried out by centrifugation (29) in a 42.1 rotor (Beckman Instruments, Inc., Fullerton, CA) followed by centrifugation through a 10–30% (vol/vol) glycerol gradient in PBS, pH 7.2 (29). Transferrin-gold resuspended in PBS containing 0.1% sodium azide was stored at 4°C. The sodium azide was removed from the transferrin-gold suspension by dialysis before use. Protein A-colloidal gold (PAG) probes were stored in 50% (vol/vol) glycerol at -20°C .

Incubation of Trypanosomes with Transferrin-Gold

Trypanosomes isolated from infected rat blood were washed twice in RPMI 1640 to remove Percoll. After centrifugation for 3 s in an Eppendorf microfuge ($\sim g_{\text{max}}$, 15,000), the loose pellet was resuspended in fresh RPMI 1640. The trypanosomes ($\sim 1 \times 10^7$ per 1 ml) were incubated with transferrin-gold at a dilution of the gold suspension where the final absorbance was between 1.0 and 1.5 at 525 nm. Incubation was carried out at 24°C for various times up to 3 h.

Fixation, Immunocytochemistry, and Electron Microscopy

After incubation, the suspended trypanosomes were fixed in 0.5% (vol/vol) glutaraldehyde by the addition of an equal volume of 1% glutaraldehyde in 100 mM Pipes adjusted to pH 7.0 by the addition of sodium hydroxide. A higher glutaraldehyde concentration (2.5%) was used to fix trypanosomes to be embedded in epoxy resin. After 1 h of fixation the trypanosomes to be embedded in epoxy resin were pelleted, postfixed in 1% (vol/vol) osmium tetroxide, stained en bloc in 1% (wt/vol) uranyl acetate in 50 mM sodium maleate buffer (pH 5.1), dehydrated in ethanol, and embedded at 60°C in epoxy resin. After fixation in 0.5% glutaraldehyde, the trypanosomes to be used for immunocytochemistry were again pelleted, the fixative was drawn off, and the pellet resuspended in 5% (wt/vol) gelatin in PBS at 37°C. After centrifugation in an Eppendorf microfuge for 2 min, the gelatin was solidified by cooling the tube, and the embedded pellet was removed by cutting away the base of the tube. The pellet was immersed in fresh 0.5% glutaraldehyde in 100 mM Pipes buffer (pH 7.0) to fix the gelatin. Pyramid-shaped blocks, ~1 mm square at the base, were cut from the pellet and immersed in 2 M sucrose in 100 mM Pipes buffer for at least 30 min, mounted on copper studs, and frozen in liquid nitrogen. The techniques for ultramicrotomy have been described elsewhere (16, 17). Sections were cut at -10°C with an MT-2B and FTS cryoattachment (Sorvall Instruments Div., Rockville, MD). Sections were labeled by incubation with either of the primary antibodies, washed with PBS, and then incubated with protein A-colloidal gold. Double labeling followed sequences 3 and 4 of Geuze et al. (12). The protein A with the smaller gold particle size was always applied after the primary antibody and the order of primary antibody labeling was alternated in each experiment. An additional 5 min incubation with a solution of protein A in PBS (0.1 mg/ml), and 5 × 1-min washes in PBS were performed before incubation with the second antibody. All antibodies and protein A gold probes were diluted in PBS containing 10% (vol/vol) FBS. Incubation times were 30 min for antibodies and 15 min for PAG probes.

Controls for single-label experiments consisted of (a) incubation of MITat 1.2 sections with PBS containing 10% (vol/vol) FBS in place of rabbit IgGs, followed by PAG; (b) substitution of normal rabbit serum for the primary antibody; and (c) incubation of sections of an unrelated clone (ETat 1.3) with RvSG IgGs.

Electron microscopy was performed using an EM 10A (Carl Zeiss GmbH, Oberkochen, FRG) operated at 80 kV.

Light Microscopy

Aliquots of 100- μ l amounts of fixed trypanosome suspensions incubated in transferrin-gold were removed before processing for electron microscopy. To these were added 100 μ l of PBS and 200 μ l of normal rat serum, and samples of each were centrifuged onto glass slides in a cytocentrifuge (Cytospin; Shandon Scientific, Runcorn, England) at 1,000 rpm for 10 min. The slides were quickly placed into water, washed three times in water and in-

cluded at 24°C in IntenSe for 5 min. The slides were fixed as recommended in the IntenSe instruction sheet and washed in water. The slides were examined with an ICM405 inverted light microscope (Carl Zeiss GmbH) equipped with $\times 63$ and $\times 100$ Planachromat, oil immersion objectives, and Nomarsky differential interference contrast illumination. All micrographs were taken on Panatomic-X (Eastman Kodak Co.) black-and-white film using the $\times 100$ objective and Nomarsky illumination.

Micrographs

Unless stated otherwise all the electron micrographs presented were from thawed, thin cryosections of trypanosomes, indirectly labeled with PAG. All the micrographs presented were chosen as representative samples of a much greater body of work from which the conclusions of this report were drawn. Unless stated otherwise the micrographs are of *T. brucei* (MITat 1.2).

Results

Endocytosis of Transferrin-Gold

When MITat 1.2 trypanosomes isolated from rat blood were incubated in suspensions of transferrin-gold they retained their motility for up to 5 h at 24°C without a change of medium. Cytocentrifuge smear preparations of fixed trypanosomes incubated for various times in transferrin-gold and incubated in IntenSe were seen to contain silver deposits inside the cells between the posteriorly placed flagellar pocket and the nucleus. Silver deposits were rarely seen on the anterior side of the nucleus. Cells not incubated in transferrin-gold did not contain this silver deposit (Fig. 1 A). A 10-min incubation in gold marker caused only small amounts of silver to be deposited (Fig. 1 B). The maximum extent of silver deposition seemed to be reached after 30 min-incubation in transferrin-gold (Fig. 1 C) with little change either in the localization or amount deposited after 80 min (Fig. 1 D) or 3 h (Fig. 1 E). The structures revealed by this silver intensification method seemed to be discrete vesicles (Fig. 1 C) and large interconnected structures (Figs. 1, C, D, and E). Silver deposits were seen in trypanosomes that were in various stages of division (Fig. 1 C). Samples of cells examined by light microscopy were also examined with the electron microscope without silver intensification. Transferrin-gold was first seen to bind to the cell surface with large

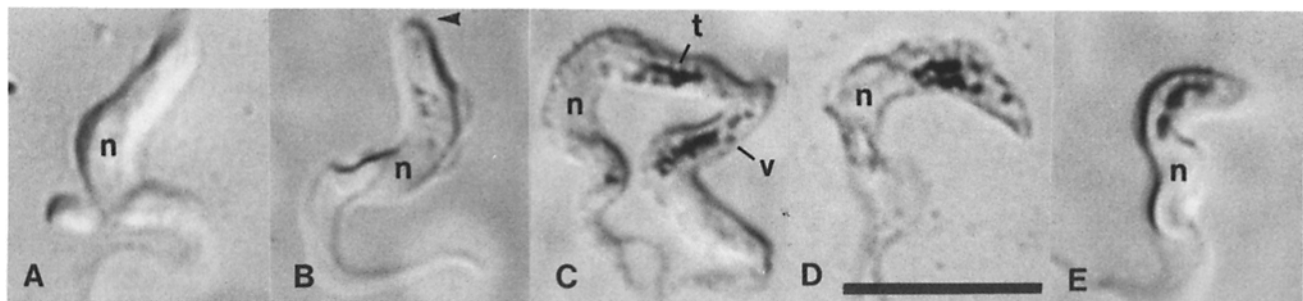


Figure 1. Light micrographs of cytocentrifuge smear preparations of whole glutaraldehyde-fixed *T. brucei* MITat 1.2 which had been incubated before fixation for various times in transferrin-gold, which was subsequently silver enhanced by IntenSe. A trypanosome not incubated in transferrin-gold but subjected to the silver enhancement procedure showed no evidence of silver deposits (A). After a 10-min incubation in transferrin-gold (B) small silver deposits were found in the region between the flagellar pocket (arrowhead) and the nucleus (n). By 30 min (C) larger silver deposits were seen on the posterior side of the nucleus (n). They were in vesicle-like structures (v) and long interconnected structures (t). The two trypanosomes in this micrograph are probably in the final stages of cellular division. After 80 min (D) and 180 min (E) silver deposits were still posterior to the nucleus (n) and in similar structures to those seen after 30-min incubation in transferrin-gold. Bar, 10 μ m.

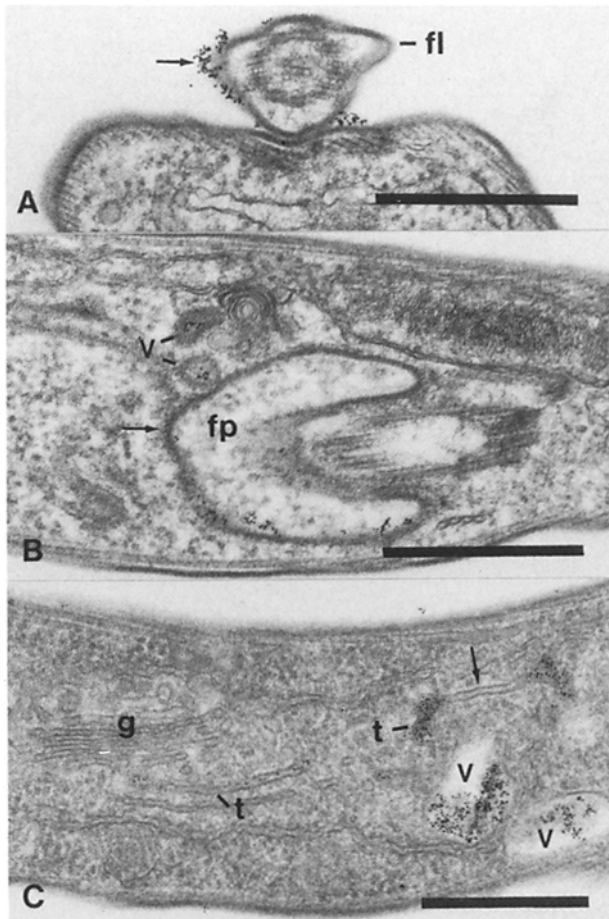


Figure 2. Electron micrographs of epoxy resin sections of *T. brucei* MITat 1.2 incubated with transferrin-gold (particle size, 5 nm). (A) *T. brucei* incubated in transferrin-gold bound the marker (arrow) in large amounts on areas of the flagellum (fl). The gold particles used were not of a homogeneous size range. (B) Uptake of transferrin-gold by *T. brucei* was via the flagellar pocket (fp) where it entered the cell via coated pits (arrow) and vesicles (v). (C) If transferrin-gold (5 nm particle size) was incubated with *T. brucei* for 5 min, tubules (t), some in close proximity to the Golgi apparatus (g), and large vesicular structures (v) filled with transferrin-gold. Note that transferrin-gold did not completely fill the tubule arrowed. Bars, 0.5 μ m.

amounts being found around the flagellum (Fig. 2 A). This binding to the cell surface and uptake into the flagellar pocket and coated vesicles (Fig. 2 B) was almost completely inhibited when trypanosomes were incubated in medium containing transferrin-gold and 5 mg/ml free bovine transferrin (not shown). Uptake of transferrin-gold was rapid and by 5 min the flagellar pocket, coated vesicles, small and large tubular structures (Fig. 2 C), and lysosomelike structures contained the marker. Tubular structures close to the Golgi apparatus also contained transferrin-gold after 5 min incubation (Fig. 2 C).

Labeling with RxVSG and RxCRD

The RxVSG IgG preparation bound to living MITat 1.2 trypanosomes as determined by indirect fluorescent antibody

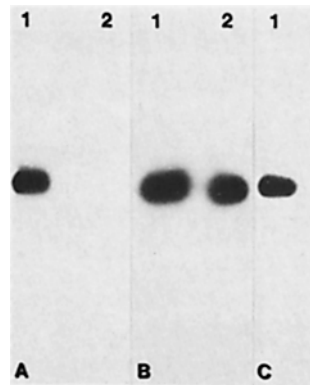


Figure 3. Immunoblot analysis of RxVSG and RxCRD IgG preparations. (A) The RxVSG IgG reacted with sVSG of *T. brucei* MITat 1.2 (lane 1) but not with sVSG of *T. brucei* MITat 1.52 (lane 2). (B) The RxCRD IgG reacted with the sVSGs of *T. brucei* MITat 1.52 (lane 1) and *T. brucei* MITat 1.2 homogenates (lane 2). (C) The RxCRD IgG also reacted with sVSG on an immunoblot of homogenized *T. congolense* ILNat 2.1 organisms (lane 1).

labeling. RxCRD IgG did not label living trypanosomes. RxVSG IgG only bound to MITat 1.2 VSG, whereas RxCRD reacted with the VSGs of other *T. brucei* clones as well as *T. congolense* on immunoblots (Fig. 3) or cryosections (data not shown).

The determinants recognized by the two IgG preparations form part of the same glycoprotein in its surface configuration, and in single-label experiments on thawed thin cryosections both IgG preparations produced similar labeling patterns. Double-label experiments revealed a close association between the determinants recognized by both antibodies on the cell surface and in many membrane-bound organelles.

The RxVSG IgG labeled the cell and flagellar surfaces of *T. brucei* MITat 1.2. The whole of the flagellar surface (Fig. 4 A), including that surface in close apposition to the cell body entering the flagellar pocket (not shown), and the membrane and luminal contents of the flagellar pocket labeled with RxVSG (Fig. 4 B). A similar labeling pattern of the cell surface and flagellar pocket was observed for the RxCRD IgG in single- and double-label experiments (not shown). Although labeling of the Golgi apparatus by both antibodies was variable it was frequently observed that the RxVSG antibody labeled all the cisternae of the Golgi apparatus (Fig. 4 C). The RxCRD antibody label was generally restricted to one side of the Golgi apparatus (Fig. 4, D and E). However, some Golgi profiles were seen that either had RxCRD label over all the stacks or had low levels of RxCRD label associated with them.

Although occasional gold particles were seen over some endoplasmic reticulum-like profiles, no strong or regular association with this organelle was observed for either antibody. Neither antibody caused labeling of the nucleus or nuclear envelope.

Labeling of Trypanosomes Containing Endocytosed Transferrin-Gold with RxVSG and RxCRD

Structures involved with endocytosis of transferrin-gold also labeled with both antibodies. The RxVSG (not shown) and RxCRD antibodies labeled transferrin-gold containing pits and vesicles which seemed to have budded from the flagellar pocket membrane (Fig. 4 D). Transferrin-gold containing tubular profiles (Fig. 4 D), vesicles, and lysosomelike structures into which vesicles seemed to be discharging their contents, labeled with RxCRD (Fig. 5 A). The RxVSG labeled similar structures in single-label experiments (not shown).

On sections of trypanosomes incubated for 3 h in transfer-

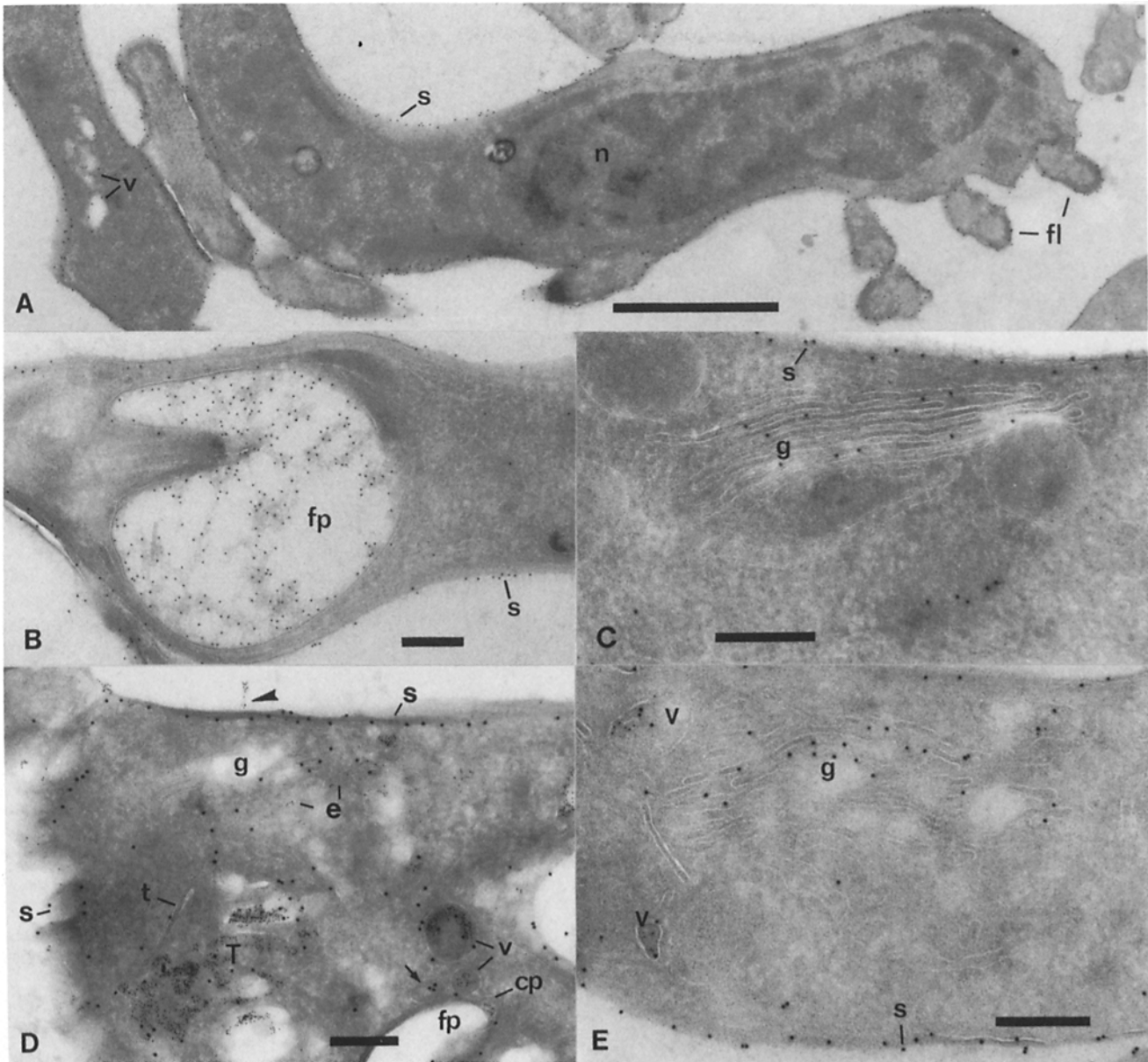


Figure 4. Antibody labeling of *T. brucei* incubated in 5 nm transferrin-gold before fixation. (A) The RxVSG antibody (8 nm PAG) bound mostly to the trypanosome surface (*s*) and the surface of the flagellum (*fl*). Labeling of other structures was negligible and the only internal labeling present was that associated with vesicles (*v*) some of which contained endocytosed transferrin-gold (after a 3-h incubation). No RxVSG label was seen over the nucleus (*n*). (B) The flagellar pocket (*fp*), both in the lumen and on the membrane surface, was labeled with RxVSG antibody (8 nm PAG). Antibody associated label was seen on the cell surface (*s*). (C) The RxVSG antibody label (8 nm PAG) was distributed over most of the Golgi cisternae profiles (*g*). Label was associated with cell surface (*s*). No endocytosed transferrin-gold particles could be seen associated with the Golgi apparatus (3-h incubation). (D) Trypanosome incubated for 10 min in transferrin-gold (5 nm particle size). The RxCRD label (10 nm PAG) was associated with transferrin-gold-containing vesicles (*v*) and pit (*cp*) associated with the flagellar pocket (*fp*) membrane. Other transferrin-gold containing structures, including a tubular network (*T*) labeled with RxCRD. The RxCRD antibody labeled one half of the Golgi apparatus (*g*), a tubular profile (*t*), and a vesicle (*arrow*) which were all free of endocytosed marker although close to the transferrin-gold containing structures. The half of the Golgi apparatus nearest to transferrin-gold-containing structures (*e*) labeled with RxCRD. The cell surface (*s*) labeled with RxCRD and had transferrin-gold bound to it (*arrowhead*). (E) Trypanosome incubated for 3 h in transferrin-gold. The RxCRD label was present over one side of the Golgi apparatus (*g*). No endocytosed gold particles could be seen in the Golgi apparatus even though transferrin-gold containing vesicles (*v*) that labeled with RxCRD were in close proximity. The RxCRD labeled the cell surface (*s*). Bars: (A) 1 μ m; (B–E) 0.2 μ m.

rin-gold before fixation and double labeled with RxVSG and RxCRD, both antibodies labeled similar sites on the cell surface and organelles involved with transferrin-gold endocytosis (Fig. 5, B and C). Tubular networks containing transfer-

rin-gold labeled with both antibodies (Fig. 5 B) as did lysosomelike structures containing transferrin-gold (Fig. 5, B and C). Often in close proximity to these transferrin-gold-containing organelles were tubules and vesicles

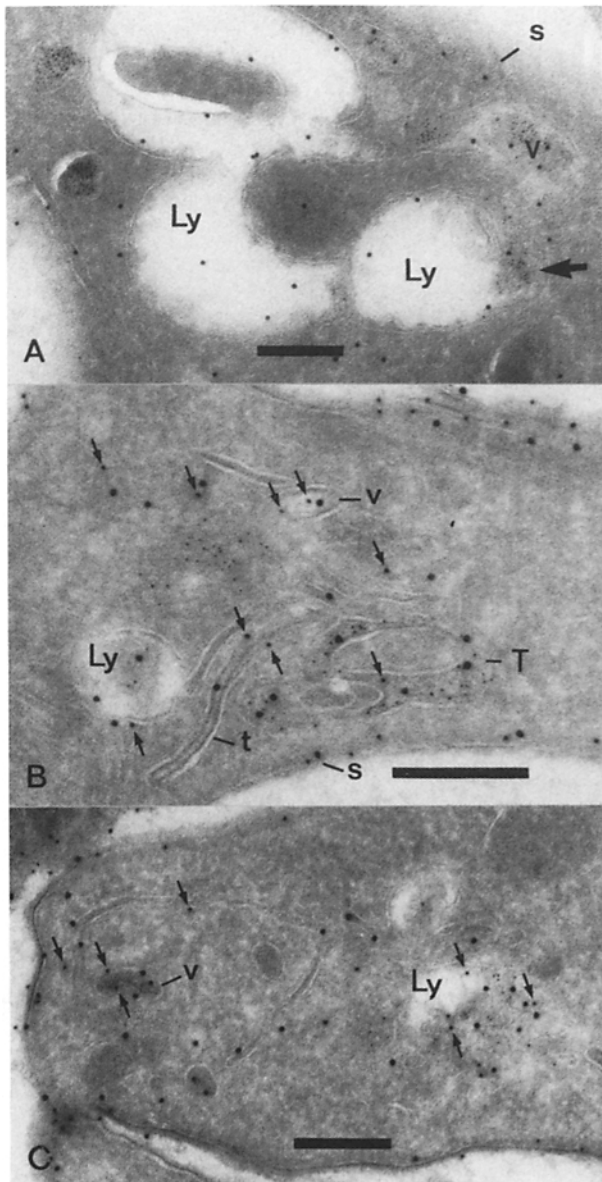


Figure 5. (A) Trypanosome incubated for 10 min in 5 nm transferrin-gold, sectioned, and then labeled with RxCRD and 15-nm particle size PAG. The RxCRD labeled the cell surface (*s*) and transferrin-gold-containing vesicles (*v*), one of which (*arrow*) seems to have fused with a lysosomelike structure (*Ly*) also labeled with RxCRD. (B) Trypanosome incubated for 3 h in 5 nm transferrin-gold and labeled with RxVSG (8 nm PAG) and RxCRD (15 nm PAG). Endocytosed transferrin-gold was present in a tubular network (*T*) that labeled with RxVSG and with RxCRD. A lysosomelike structure (*Ly*) containing transferrin-gold also labeled with both antibodies. Tubules (*t*) and a vesicle (*v*) labeled with both antibodies but did not contain transferrin-gold. Intracellular RxVSG label is arrowed. Both antibodies labeled the cell surface (*s*). (C) A section from a similar preparation to that in (B). A lysosomelike structure (*Ly*) containing endocytosed transferrin-gold (5 nm particle size) was labeled with RxVSG (8 nm PAG some are arrowed) and RxCRD (15 nm PAG). A vesicle (*v*) and tubular profiles not containing transferrin-gold labeled with RxVSG (*arrows*) and RxCRD. Bars, 0.2 μm .

that labeled with both antibodies but did not contain endocytosed gold (Fig. 5, B and C). In trypanosomes incubated for different times in transferrin-gold, tubules not containing endocytosed gold were seen to have transferrin-gold containing vesicles attached to their lumen. In single-label experiments the tubules free of endocytosed gold, but contiguous with transferrin-gold-containing vesicles, labeled with RxVSG (Fig. 6 A) and RxCRD (Fig. 6 B). In the double-labeling experiments similar structures labeled with both antibodies (Fig. 6 C).

Possible Segregation of VSG from the CRD

Evidence for segregation of the determinants recognized by RxVSG from those recognized by RxCRD was revealed by double-labeling experiments with both antibodies. Tubular structures close to the flagellar pocket labeled strongly with RxCRD compared with the RxVSG label. The RxVSG antibody labeled only one side of these tubular structures (Fig. 6, D, E, and F), whereas the RxCRD label was either over the whole profile (Fig. 6, D and E) or was separately situated within the structure (Fig. 6 F). In trypanosomes incubated for 3 h in transferrin-gold before fixation the tubular structures did not contain endocytosed marker (Fig. 6, D and E).

The differential labeling occurred regardless of the sequence of addition of antibody, i.e., regardless of whether RxCRD (Fig. 6 D) or RxVSG (Fig. 6 E) were applied first and labeled with the smaller PAG particle size.

Experimental Controls

When RxVSG IgG preparations were used to label sections of *T. brucei* (ETat 1.3), a clone expressing an unrelated VSG, very low levels of labeling with PAG were produced (Fig. 7 A). This was also the case when sections of *T. brucei* (MITat 1.2) were incubated during the staining procedures with either 10% FBS (Fig. 7 B) or normal rabbit serum (Fig. 7 C) in place of primary antibody. Few PAG particles were seen on sections and they were randomly distributed. Some batches of PAG when used alone selectively labeled the nucleolus of trypanosome nuclei, and in some cases, the kinetoplast. These were discarded in favour of PAG that produced low, random background labeling even when used at high concentrations.

Discussion

In this report VSG has been immunocytochemically localized at sites other than the cell surface, and the cointernalization of VSG with transferrin-gold marker has been demonstrated. Organelles involved with endocytosis of transferrin-gold particles, the flagellar pocket, and other cytoplasmic structures not obviously involved with endocytosis have all been shown to contain VSG and CRD. Tubular profiles not containing endocytosed gold marker but connected to endocytotic organelles have also been shown to label with RxVSG and RxCRD. A more comprehensive description of the organelles involved with endocytosis of ferritin in trypanosomes made by Langreth and Balber (25) described a tubular network opening out into flattened cisternae. The organelles involved with transferrin-gold uptake are morphologically similar to those involved with ferritin uptake. In mammalian cell systems transferrin-gold markers are delivered to lysosomes (26, 32). It is therefore possible

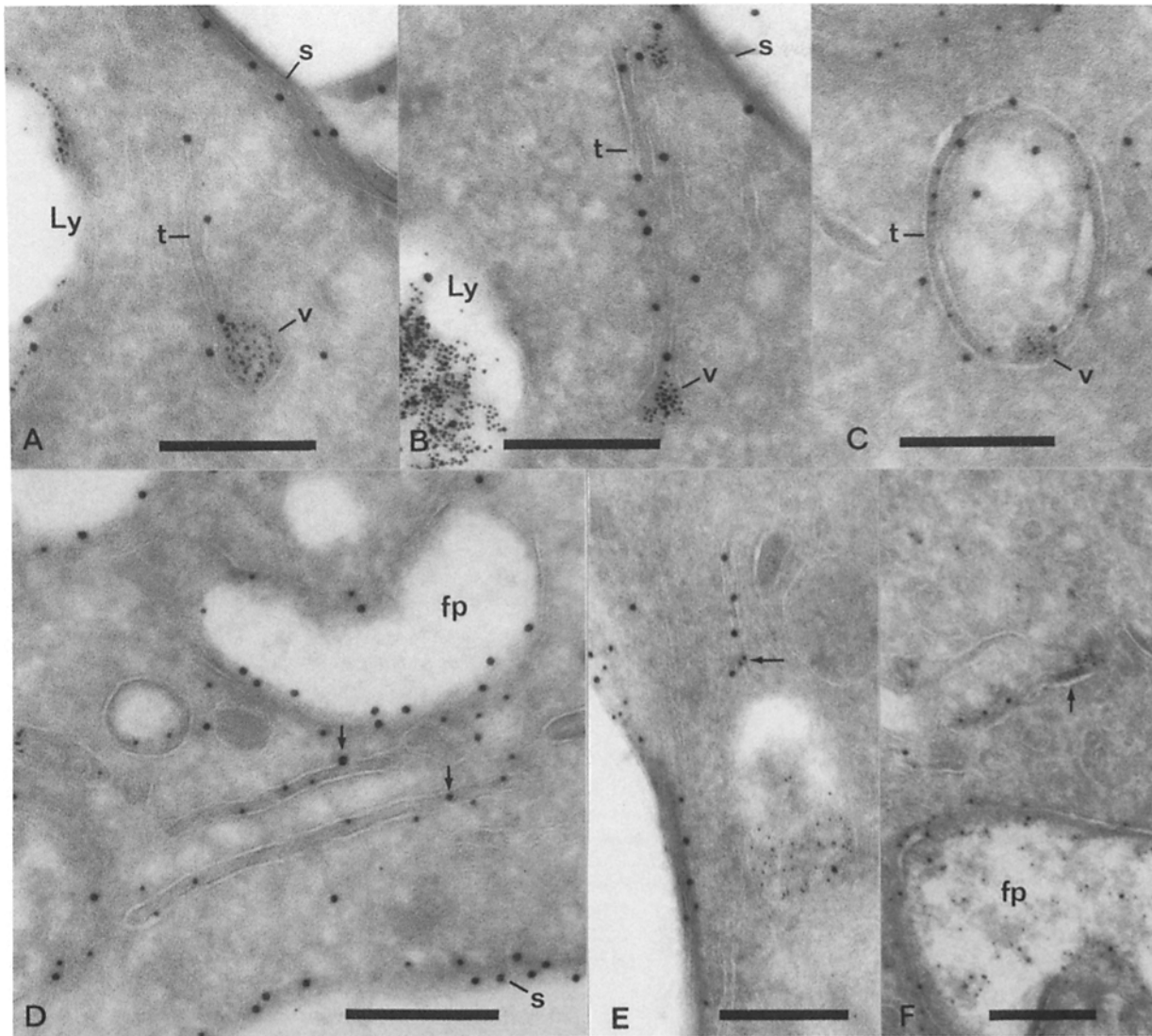


Figure 6. (A) A tubular structure (*t*) in a trypanosome incubated with 5 nm transferrin-gold for 30 min is connected to a vesicle (*v*) that contains transferrin-gold. The tubule labeled with RxVSG (15 nm PAG) but did not contain transferrin-gold. The cell surface (*s*) and a lysosomelike structure (*Ly*) that contains transferrin-gold, labeled with RxVSG. (B) A similar tubular structure (*t*) connected to a transferrin-gold-containing vesicle (*v*) in a trypanosome incubated for 3 h in 5 nm transferrin-gold did not contain endocytosed gold but labeled with RxCRD (15 nm). A lysosomelike structure (*Ly*) containing transferrin-gold labeled with RxCRD. Label was present on the cell surface (*s*). (C) A trypanosome incubated for 1 h in 5 nm transferrin-gold and double labeled with RxVSG (8 nm PAG) and RxCRD (15 nm PAG) had a transferrin-gold-containing vesicle (*v*) open on two sides to a tubular structure (*t*) which labeled with both antibodies but did not contain transferrin-gold. (D) Two tubular structures close to the flagellar pocket (*fp*) label with RxCRD (8 nm PAG). The amount of RxVSG label (15 nm PAG) in these structures was low (*arrows*) even though the flagellar pocket (*fp*) and cell surface (*s*) were well labeled with RxVSG. The trypanosome was incubated for 1 h in 5 nm transferrin-gold but this is not present in the flagellar pocket or the labeled tubules. (E) A trypanosome incubated in 5 nm transferrin-gold for 1 h and double labeled with RxVSG (8 nm PAG) and RxCRD (15 nm PAG). A tubule that did not contain transferrin-gold labeled with RxCRD. The RxVSG label (*arrow*) was restricted to one end. The cell surface (*s*) was labeled by both antibodies and a transferrin-gold-containing structure was labeled with RxCRD. (F) This cell not incubated in transferrin-gold is labeled with RxCRD (5 nm PAG) and RxVSG (10 nm PAG). A tubular profile was labeled on one side with RxCRD (*arrow*) and on the other side with RxVSG. The flagellar pocket (*fp*) was labeled by both antibodies. Bars, 0.2 μm .

that some of the transferrin-gold containing organelles in this study may represent trypanosomal lysosomes. This can only clearly be shown when distinct markers for these organelles in trypanosomes become available.

Endocytosed gold particles were visualized for light microscopy by silver enhancement. Using this method the organelles involved with transferrin-gold uptake in *T. brucei*

were localized between the flagellar pocket and the nucleus of the cell. Many of the larger structures appeared to interconnect and the structures visualized by the silver enhancement treatment did not appear to change their position in the cell after longer incubation times.

Serial sectioning studies have revealed that many of the transferrin-gold containing tubular profiles are in fact sec-

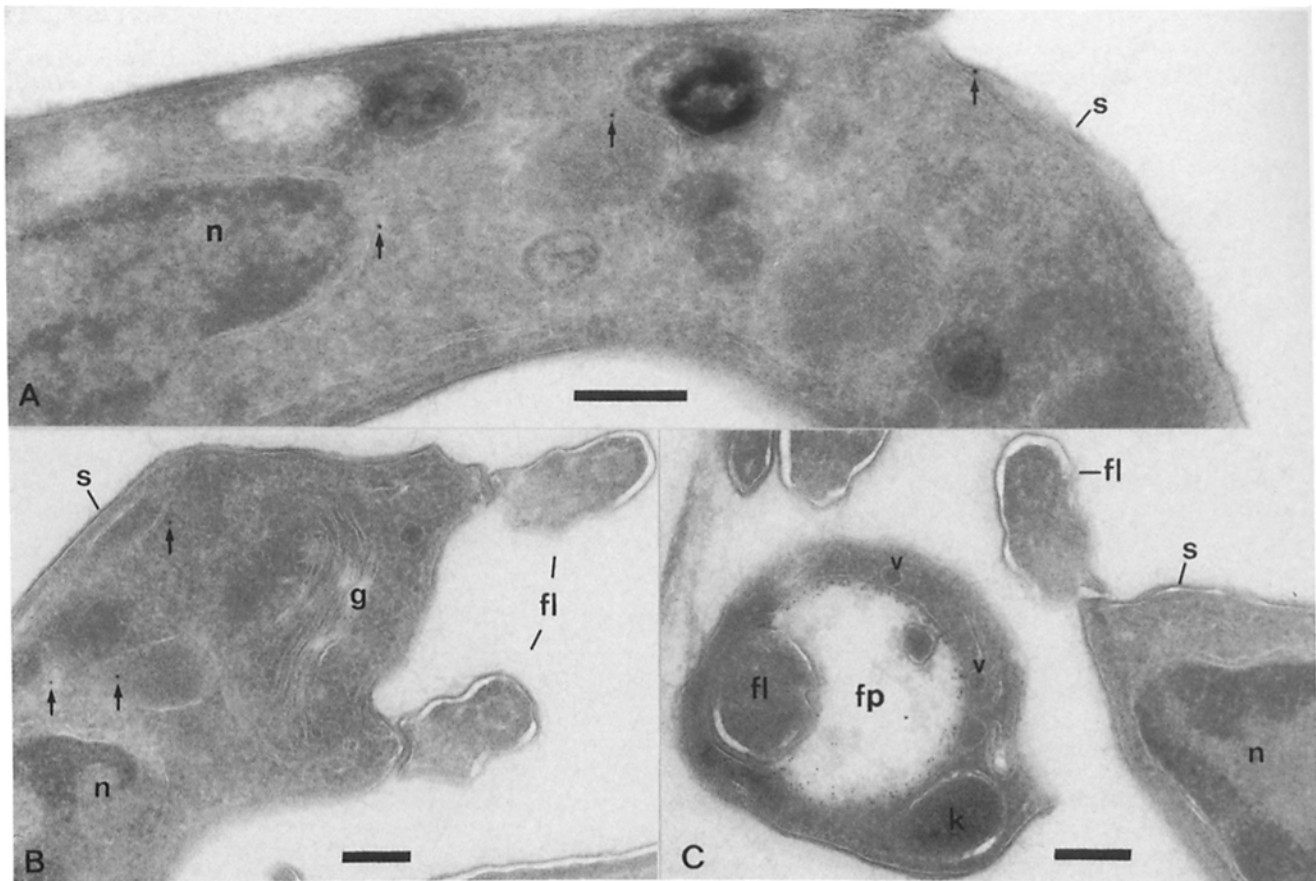


Figure 7. Representative sections from control experiments. (A) *T. brucei* (clone ETat 1.3) section incubated with RxVSG IgG and 8 nm PAG. No specific labeling was apparent. Three gold particles (arrows) were present on the section but only one was associated with the surface (s). No gold particles were associated with the nucleus (n). (B) Control using normal rabbit serum in place of primary antibody on trypanosomes incubated for 3 h in transferrin-gold. Three 8-nm PAG particles were present (arrows) but no label was seen associated with the Golgi apparatus (g), the cell surface (s), the flagellum (fl), or the nucleus (n). No transferrin-gold was seen in association with the Golgi apparatus. (C) Control using 10% (vol/vol) FBS in PBS substituted for the primary antibody, followed by 8 nm PAG on trypanosomes incubated for 3 h in 15 nm transferrin-gold before fixation. Transferrin-gold was present inside the flagellar pocket (fp) in association with the membrane. Two vesicles containing transferrin-gold could also be seen (v). There were no 8-nm PAG particles present on the cell surface, the nucleus (n), flagellum (fl), or kinetoplast (k). Bars, 0.2 μm .

tions through cisternae (Webster, P., unpublished observations). Because serial sections are not shown in the present study, for the sake of simplicity these structures are referred to here as tubules.

The inhibition of both the binding of transferrin-gold to the surface and the flagellar pocket of *T. brucei*, and the inhibition of its uptake by the presence of excess free transferrin suggests that the cell surface and the flagellar pocket membrane may contain receptors for transferrin. If receptors for transferrin are present on the trypanosome cell surface and flagellar pocket membrane and differ from their mammalian host, then they could form the basis of a new approach to vaccination against animal trypanosomiasis.

The presence of RxCRD and RxVSG label over the flagellar pocket membrane is in agreement with previous observations of a VSG-like structure on this membrane (22, 25) in epoxy resin sections and with immunofluorescence studies (2a). However, the antibody labeling was not confined to the flagellar pocket membrane but was often evenly distributed throughout the lumen of the flagellar pocket.

The presence of tubules that labeled with both antibodies and were connected to transferrin-gold-containing vesicles but which did not contain any transferrin-gold, suggests trypanosomes may possess a mechanism for excluding exogenous proteins from part of the endocytosis pathway.

The VSG determinants recognized by the two antibodies seem to be sorted from each other in tubular structures, similar to those connected to transferrin-gold-containing structures that labeled with both antibodies but did not contain transferrin-gold. Such segregation suggests that the different components of the VSG may be required and metabolized in different sites in the parasite.

Many vesicles that labeled with both antibodies but did not contain endocytosed transferrin-gold were observed inside actively endocytosing trypanosomes. This suggests that these structures may be involved in VSG recycling and/or transport of newly synthesized VSG from the Golgi complex to the cell surface. These vesicles were often found in close proximity to the flagellar pocket and to other vesicles which contained large amounts of gold marker.

It is surprising that the CRD antibody can be used in these studies to localize CRD in the trypanosome, for it is reported that this ligand is recognized by antibody only after it has been modified by the mfVSG phospholipase (6, 10). However, it appears that glutaraldehyde may cause conformational changes in mfVSG, exposing the CRD to the antibody (Fish, W. R., and D. J. Grab, unpublished observation). Thus the chemical fixation used in these studies may have caused the CRD to be revealed on mfVSG inside the cell and on the cell surface. The unpublished results (Turner, M. J., quoted in reference 23) showing that CRD antibody will still bind mfVSG offer another explanation for RxCRD antibody labeling. In addition, the high levels of mfVSG phospholipase levels in isolated Golgi and flagellar pocket/endosome fractions as compared with levels in rough endoplasmic reticulum fractions (14) may be responsible for modifying mfVSG in these organelles to forms recognized by the CRD antibody thus explaining RxCRD binding on cryosections of trypanosomes.

The low levels of labeling by both antibodies over rough endoplasmic reticulum profiles suggests that both antigens are in relatively low concentrations in this structure. For both, there were significantly higher levels of labeling over the cisternae of the Golgi apparatus. Whereas the RxVSG was found uniformly over the Golgi cisternae the RxCRD labeling was restricted to cisternae on one side of the Golgi apparatus. This is consistent with previous studies (15). We postulate that the CRD may be added to the VSG molecule in the Golgi apparatus or the VSG molecule is further modified in the Golgi apparatus to cause the CRD to be recognized by the antibody at this site.

The pathways of endocytosis of transferrin-gold seem morphologically similar to the pathways taken by other markers in trypanosomes (25). Using antibodies to parts of the VSG molecule, we have demonstrated that the tubular networks involved in endocytosis in trypanosomes are able to segregate VSG molecules from transferrin-gold that has been endocytosed. In addition, segregation of the CRD from the rest of the VSG molecule has been shown to occur in similar tubular profiles that also did not contain endocytosed transferrin-gold. Perhaps the two processes of VSG segregation and degradation are sequential and occur in interconnected organelles. Although prelysosomal and lysosomal compartments have not been identified morphologically in trypanosomes, it seems that some parts of the tubular networks involved with transferrin-gold endocytosis have similar molecular sorting functions to the prelysosomal and/or endosome compartments of mammalian cells and can be justifiably referred to as endosomes.

The ICM 405 light microscope and accessories used in this study were kindly loaned by Carl Zeiss Oberkochen and were made available by Achelis (Kenya) Limited. Mr. E. G. Okoth of Achelis (Kenya) Limited allowed the light microscopy studies to be completed in his offices. Drs. P. R. Gardiner, G. Griffiths, and J. D. Lonsdale-Eccles provided constructive criticisms during preparation of the manuscript. Trypanosomes were isolated from infected blood and made available for our use by Dr. S. Z. Shapiro (*T. brucei* ETat 1.3), Mrs. Y. Verjee, and Mr. S. Wasike (*T. brucei* MITat 1.2 and all other trypanosome populations). P. T. Englund, R. Bülow, J. W. Slot, and G. Griffiths generously provided copies of manuscripts before publication. Ms. Doris Churi, and Ms. H. Walji provided secretarial assistance. This is ILRAD publication No. 495.

Received for publication 7 July 1987.

References

- Bangs, J. D., D. Herald, J. L. Krakow, G. W. Hart, and P. T. Englund. 1985. Rapid processing of the carboxyl terminus of a trypanosome variant surface glycoprotein. *Proc. Natl. Acad. Sci. USA.* 82:3207-3211.
- Barbet, A. F., and T. C. McGuire. 1978. Crossreacting determinants in variant-specific surface antigens of African trypanosomes. *Proc. Natl. Acad. Sci. USA.* 75:1989-1993.
- Barry, J. D., and K. Vickerman. 1979. *Trypanosoma brucei*: loss of variable antigens during transformation from bloodstream to procyclic forms *in vitro*. *Exp. Parasitol.* 48:313-324.
- Brown, K. N., J. A. Armstrong, and R. C. Valentine. 1965. The ingestion of protein molecules by blood forms of *Trypanosoma rhodesiense*. *Exp. Cell Res.* 29:129-139.
- Bülow, R., and P. Overath. 1985. Synthesis of a hydrolase for the membrane-form variant surface glycoprotein is repressed during transformation of *Trypanosoma brucei*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 187:105-109.
- Burnette, W. N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.
- Cardoso de Almeida, M. L., and M. J. Turner. 1983. The membrane form of variant surface glycoproteins of *Trypanosoma brucei*. *Nature (Lond.)*. 302:349-352.
- Cardoso de Almeida, M. L., L. M. Allan, and M. J. Turner. 1984. Purification and properties of the membrane form of variant surface glycoproteins (VSGs) from *Trypanosoma brucei*. *J. Protozool.* 31:53-60.
- Cross, G. A. M. 1975. Identification and properties of clone-specific glycoprotein antigens constituting the surface coat of *Trypanosoma brucei*. *Parasitology.* 71:393-417.
- Cross, G. A. M. 1984. Structure of the variant glycoproteins and surface coat of *Trypanosoma brucei*. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 307:3-12.
- Ferguson, M. A. J., and G. A. M. Cross. 1984. Myristylation of the membrane form of a *Trypanosoma brucei* variant surface glycoprotein. *J. Biol. Chem.* 259:3011-3015.
- Ferguson, M. A. J., K. Halder, and G. A. M. Cross. 1985. *Trypanosoma brucei* variant surface glycoprotein has a sn-1,2-dimyristyl glycerol membrane anchor at its COOH terminus. *J. Biol. Chem.* 260:4963-4968.
- Geuze, H. J., J. W. Slot, P. van der Ley, R. T. C. Scheffer, and J. M. Griffiths. 1981. Use of colloidal gold particles in double labelling immunoelectron microscopy of ultrathin frozen tissue sections. *J. Cell Biol.* 89: 653-665.
- Grab, D. J., and J. J. Bwayo. 1982. Isopycnic isolation of African trypanosomes on Percoll gradients formed *in situ*. *Acta Trop.* 39:363-366.
- Grab, D. J., P. Webster, W. R. Fish, Y. Verjee, and J. D. Lonsdale-Eccles. 1987. Subcellular localization of a variable surface glycoprotein phosphatidylinositol-specific phospholipase-C in African trypanosomes. *J. Cell Biol.* 105:737-746.
- Grab, D. J., P. Webster, and Y. Verjee. 1984. The intracellular pathway and assembly of newly formed variable surface glycoprotein of *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. USA.* 81:7703-7707.
- Griffiths, G., A. McDowall, R. Back, and J. Dubochet. 1984. On the preparation of cryosections for immunocytochemistry. *J. Ultrastruct. Res.* 89: 65-78.
- Griffiths, G., K. Simons, G. Warren, and K. T. Tokuyasu. 1984. Immunoelectron microscopy using thin frozen sections: application to studies of the intracellular transport. Semliki Forest virus spike glycoproteins. *Methods Enzymol.* 96:466-485.
- Gurnett, A. M., J. Raper, and M. J. Turner. 1986. Solution properties of the variant surface glycoprotein of *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* 18:141-153.
- Gurnett, A. M., J. Ward, J. Raper, and M. J. Turner. 1986. Purification and characterization of membrane-form variant surface glycoproteins of *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* 20:1-13.
- Helenius, A., I. Mellman, D. Wall, and A. Hubbard. 1983. Endosomes. *Trends Biochem. Sci.* 8:245-250.
- Holder, A. A., and G. A. M. Cross. 1981. Glycopeptides from variant surface glycoproteins of *Trypanosoma brucei*. C-Terminal localisation of antigenically crossreacting carbohydrate moieties. *Mol. Biochem. Parasitol.* 2:135-150.
- Horisberger, M., and M. F. Clerc. 1985. Labelling of colloidal gold with protein A. A quantitative study. *Histochemistry.* 82:219-223.
- Jackson, D. G., and H. P. Voorheis. 1985. Release of the variable surface coat glycoprotein from *Trypanosoma brucei* requires the cleavage of a phosphate ester. *J. Biol. Chem.* 260:5179-5183.
- Klausner, R. D., G. Ashwell, J. van Renswoude, J. B. Hartford, and K. R. Bridges. 1983. Binding of apotransferrin to K 562 cells: explanation of the transferrin cycle. *Proc. Natl. Acad. Sci. USA.* 80:2263-2266.
- Langreth, S. G., and A. E. Balber. 1975. Protein uptake and digestion in bloodstream and culture forms of *Trypanosoma brucei*. *J. Protozool.* 22: 40-53.
- Neutra, M. R., A. Ciechanover, L. S. Owen, and H. F. Lodish. 1985. Intracellular transport of transferrin- and asialoosomucoid-colloidal gold

- conjugates to lysosomes after receptor mediated endocytosis. *J. Histochem. Cytochem.* 33:1134-1144.
27. Shapiro, S. Z., and T. M. Pearson. 1986. African trypanosomiasis: antigens and host-parasite interactions. In *Parasite Antigens Towards New Strategies for Vaccines*. T. M. Pearson, editor. Marcel Dekker Inc., New York. 215-274.
28. Silverstein, S. C., R. M. Steinman, and Z. A. Cohn. 1977. Endocytosis. *Annu. Rev. Biochem.* 46:699-772.
29. Slot, J. W., and H. J. Geuze. 1981. Sizing of Protein A-colloidal gold probes for immunoelectron microscopy. *J. Cell Biol.* 90:533-536.
30. Slot, J. W., and H. J. Geuze. 1985. A new method of preparing gold probes for multiple-labelling cytochemistry. *Eur. J. Cell Biol.* 38:87-93.
31. Turner, M. J. 1984. Antigenic variation in its biological context. *Phil. Trans. R. Soc. Lond. B. Biol. Sci.* 307:27-40.
32. Willingham, M. C., J. A. Hanover, R. B. Dickson, and I. Pastan. 1984. Morphologic characterisation of the pathway of transferrin endocytosis and recycling in human KB cells. *Proc. Natl. Acad. Sci. USA.* 81:175-179.