INFECTION AND IMMUNITY, Sept. 1987, p. 2137–2141
0019-9567/87/092137-05$02.00/0
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Vol. 55, No. 9

Ultrastructural Localization of an Intracellular *Toxoplasma* Protein That Induces Protection in Mice

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Received 5 December 1986/Accepted 29 May 1987

We report the ultrastructural localization of *Toxoplasma* antigens recognized by monoclonal antibody F3G3, which protects mice against lethal challenge. By using colloidal immunogold labeling, F3G3 failed to react with the surface of intact *Toxoplasma* cells, confirming previous observations that it recognizes an intracellular antigen. Immunoperoxidase labeling with F3G3 was obtained only when *Toxoplasma* cells were previously exposed to Triton X-100. A specific immunoperoxidase reaction was located beneath the surface membrane in the region of the pellicle and within the elaborate network of vesicles which are extruded from the surface of *Toxoplasma* cells during entry into host cells. Similar results were obtained with mouse polyclonal sera and a second monoclonal antibody, 6-86-1E11, both of which were produced against the F3G3 affinity-purified protein. The location of the epitope recognized by F3G3 was confirmed by purifying *T. gondii*-derived surface membrane vesicles, separating these proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting. Monoclonal antibody 6-86-1E11 and the analogous polyclonal sera reacted with the antigenically related proteins of 58 and 28 kilodaltons that were found both in the intact organisms and in the purified surface membrane vesicles. These results indicate that the epitope recognized by F3G3 is located beneath the *Toxoplasma* cell surface membrane and is contained within plasma membrane-derived vesicles.

Toxoplasma gondii is an opportunistic intracellular pathogen that contains both cell surface and internal antigens that are capable of eliciting immunological responses that partially protect mice from lethal challenge (1, 5). One such protective antigen consists of a cytoplasmic protein recognized by monoclonal antibody F3G3 (3). Mice immunized with F3G3-purified protein develop both high-titer antibody levels and elevated cell-mediated responses to *T. gondii*, which enable them to survive lethal *Toxoplasma* challenge (8). In light of previous reports that provide seemingly contradictory evidence on whether this antigen is normally exposed on viable *Toxoplasma* cells (2, 7, 8), we undertook its localization by immunoelectron microscopy and immunoblotting.

During host cell entry, *T. gondii* forms an elaborate membranous network within the host cell phagosome that contains *T. gondii*-specific surface proteins (9). Components of this *T. gondii*-derived network are present along with extracellular *Toxoplasma* cells that are isolated from host cells in the presence of calcium (9). Moreover, the networks can be purified as discrete vesicles by using a previously described system of calcium-free dispersion and differential centrifugation (9). We used this method of preparing *Toxoplasma* cells to examine the subcellular distribution of F3G3 reactivity in both intact *Toxoplasma* cells and purified network vesicles. We demonstrate here that F3G3 and an analogous monoclonal antibody, 6-86-1E11, react with two antigenically related proteins of 58 and 28 kilodaltons (kDa) that are located primarily beneath the cell surface membrane.

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MATERIALS AND METHODS

Parasite culture. *Toxoplasma gondii* RH tachyzoites were harvested from mouse ascitic fluid in Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) containing 1 mM Ca^{2+}, 10 U of heparin (Sigma Chemical Corp. St. Louis, Mo.) per ml, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.2; GIBCO). Host cells were removed by filtration through polycarbonate membrane filters (pore size, 3.0 μm; Nuclepore Corp., Pleasanton, Calif.), and *Toxoplasma* cells were suspended in phosphate-buffered saline (PBS) containing 1 mM Ca^{2+} (pH 7.2, 4°C; Irvine Scientific, Santa Ana, Calif.).

Antisera production. Monoclonal antibodies F3G3 (3, 8) and 1E11 to the major surface protein of 30 kDa (2) have been described previously. Polyclonal mouse sera were produced by injection of F3G3 affinity-purified protein (58 kDa) in Freund incomplete adjuvant (8) and is referred to here as polyclonal anti-F3G3-Ag. In addition, a second monoclonal antibody, 6-86-1E11, was produced from mice immunized with F3G3 affinity-purified antigen. Monoclonal antibody 6-86-1E11 is not related to 1E11 (2) and reacts positively with the 58-kDa F3G3-purified protein by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis. Polyvalent antisera to *T. gondii* was collected from mice chronically infected with *T. gondii* C56 that survived challenge with live *T. gondii* RH. For immunolabeling, specific antisera or normal mouse sera were diluted 1:10 to 1:50 in PBS containing 1% bovine serum albumin.

Electron microscopy. (i) Colloidal gold. Freshly isolated *Toxoplasma* cells were fixed for 10 min with 0.5% glutaraldehyde in PBS (pH 7.2) at 4°C. For surface labeling, lightly

fixed *Toxoplasma* cells were washed in PBS and incubated successively in antisera followed by washing with PBS at 4°C as follows: (i) primary antisera, 30 min; (ii) biotinylated goat anti-mouse immunoglobulin G (IgG; Bethesda Research Laboratories, Gaithersburg, Md.), 1:20 dilution, 30 min; (iii) avidin-colloidal gold (20 nm) conjugate (Bethesda Research Laboratories), 1:20 dilution, 30 min. Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and postfixed with 1% osmium tetroxide reduced with 0.5% potassium ferricyanide. Thin sections of LR White resin (London Resin Co. Ltd., Hampshire, England)-embedded specimens were stained with uranyl acetate and lead citrate and viewed with an electron microscope (EM 410; Phillips).

(ii) Immunoperoxidase. Freshly isolated *Toxoplasma* cells were fixed for 20 min with 2% formaldehyde–0.5% glutaraldehyde in 0.1 M cacodylate buffer at 4°C. Some specimens were extracted with 0.2% Triton X-100 during fixation. Lightly fixed cells were incubated in diluted primary antisera for 30 min at 4°C, washed, and incubated in a 1:20 dilution of peroxidase-conjugated goat [IgG-F(ab')$_2$] anti-mouse IgG (Tago, Inc., Burlingame, Calif.) for 30 min at 4°C. Cells were then refixed in 2.5% glutaraldehyde for 1 h at 4°C and rinsed in 0.1 M Tris hydrochloride (pH 7.5), and peroxidase activity was developed for 3 min in 0.05% diaminobenzidine (Polysciences Inc., Warrington, Pa.) containing 0.006% H_2O_2. Cells were postfixed in potassium ferricyanide-reduced osmium. LR White resin sections were examined without further staining.

SDS-PAGE immunoblotting. *T. gondii*-derived networks were purified as vesicles by sedimentation at 100,000 × *g* after dissociation from intact *Toxoplasma* cells in calcium-free buffer as described previously (9). *Toxoplasma* network vesicles or whole *Toxoplasma* antigen were prepared for electrophoresis by boiling in running buffer containing 1% SDS and 2% 2-mercaptoethanol. Whole *Toxoplasma* lysate and purified network vesicles were loaded at 20 and 5 μg of protein per lane, respectively. Proteins were separated in 6 to 14% gradient acrylamide gels by the method described by Laemmli (6) and silver stained as described previously (9). Molecular weight markers were as follows: cytochrome *c* (12,300), lysozyme (14,000), α-chymotrypsin (25,700), ovalbumin (43,000), bovine serum albumin (68,000), phosphorylase *b* (97,400), and myosin (200,000) (Bethesda Research Laboratories).

SDS-PAGE-separated proteins were electrophoretically transferred to nitrocellulose paper (pore size, 0.2 μm; Schleicher & Schuell, Inc., Keene, N.H.) by using the buffer system described by Towbin et al. (10). Nitrocellulose paper strips were preblocked for 1 h with PBS containing 1% Tween 80 and 3% bovine serum albumin, and washed in PBS containing 0.1% Tween 80. Preblocked nitrocellulose paper strips were then incubated with primary antisera diluted in PBS containing 1% bovine serum albumin for 1 h at 37°C, washed in PBS containing 1% bovine serum albumin, and labeled with ^{125}I-labeled protein A (1 × 10^6 cpm/ml; 2 × 10^6 cpm/μg of protein). After extensive washing in PBS, nitrocellulose paper strips were air dried and autoradiographed with film (XAR-2; Eastman Kodak Co., Rochester, N.Y.).

RESULTS

Colloidal gold surface labeling. To demonstrate surface labeling of *Toxoplasma* cells, monoclonal antibody 1E11, which reacts with the major surface protein of 30 kDa (2), and polyclonal chronic mouse sera were selected as positive controls. Lightly fixed *Toxoplasma* cells were reacted with primary antisera and then secondarily labeled with biotinylated goat anti-mouse IgG followed by labeling with avidin-colloidal gold. *Toxoplasma* cells incubated with control mouse sera exhibited minimal binding of colloidal gold (Fig. 1a). In contrast, chronically infected mouse sera (Fig. 1b) and 1E11 (Fig. 1c)-treated *Toxoplasma* cells were both strongly labeled with colloidal cold. There was no significant binding of colloidal gold to *Toxoplasma* cells treated with F3G3 (Fig. 1d) or polyclonal anti-F3G3-Ag sera (not shown), however, indicating that this antigen is not exposed on the surface of intact *Toxoplasma* cells.

Immunoperoxidase. To demonstrate surface versus intracellular localization, immunoperoxidase labeling was compared on lightly fixed *Toxoplasma* cells that were either intact or extracted with Triton X-100. Normal mouse sera showed a minimal background immunoperoxidase reaction product (Fig. 2a), whereas sera from mice chronically infected with *T. gondii* gave a strongly positive reaction primarily at the surface of the organisms (Fig. 2b). No significant differences in immunoperoxidase labeling by chronically infected or normal mouse sera were evident between intact versus extracted *Toxoplasma* cells. In contrast, Triton X-100 extraction was necessary for positive immunoperoxidase labeling with F3G3. Minimal labeling occurred with F3G3 on intact but lightly fixed *Toxoplasma* cells (Fig. 2c). After Triton X-100 treatment, a positive immunoperoxidase reaction with F3G3 was evident as a continuous layer around the organism (Fig. 2d). In addition, a positive immunoperoxidase reaction on Triton X-100-treated samples labeled with F3G3 was evident in membrane vesicles associated with *Toxoplasma* cells that were purified in the presence of 1 mM Ca^{2+} (Fig. 2d). A similar reaction pattern was observed with 6-86-1E11 and polyclonal anti-F3G3-Ag sera (data not shown).

Immunoblotting. To confirm that the F3G3-reactive epitope was found within *T. gondii*-derived networks, we purified the network vesicles and analyzed their protein content by silver staining and immunobloting. F3G3 was not used for immunoblotting because of its very weak affinity for SDS-PAGE-separated antigen. Instead, 6-86-1E11 and polyclonal anti-F3G3-Ag sera, which react with the F3G3 affinity-purified 58-kDa antigen after SDS-PAGE, were used for immunoblotting. In SDS-PAGE-separated whole *Toxoplasma* lysate, both the polyclonal anti-F3G3-Ag sera and 6-86-1E11 labeled two proteins of 58 and 28 kDa (apparent molecular mass) (Fig. 3). The 28-kDa protein migrated below the predominant 30-kDa protein observed in silver-stained gels of the purified network vesicles (Fig. 3). The 58- and 28-kDa proteins represent minor components of the networks and were only visible as faint bands in silver-stained gels when they were intentionally overdeveloped (data not shown). In addition, both the 58- and 28-kDa proteins were specifically labeled in *T. gondii*-derived network vesicles by 6-86-1E11 and polyclonal anti-F3G3-Ag sera but not by normal mouse sera (Fig. 3). In the purified network vesicles the reactivity of the 28-kD protein with both 6-86-1E11 and polyclonal anti-F3G3-Ag sera was predominant (Fig. 3).

DISCUSSION

In contrast to chronically infected mouse sera and 1E11, which recognize externally exposed antigens, F3G3 and polyclonal anti-F3G3-Ag sera failed to bind externally to intact *Toxoplasma* cells. We have shown ultrastructurally, using both colloidal gold and immunoperoxidase labeling that the F3G3 epitope is not exposed on lightly fixed

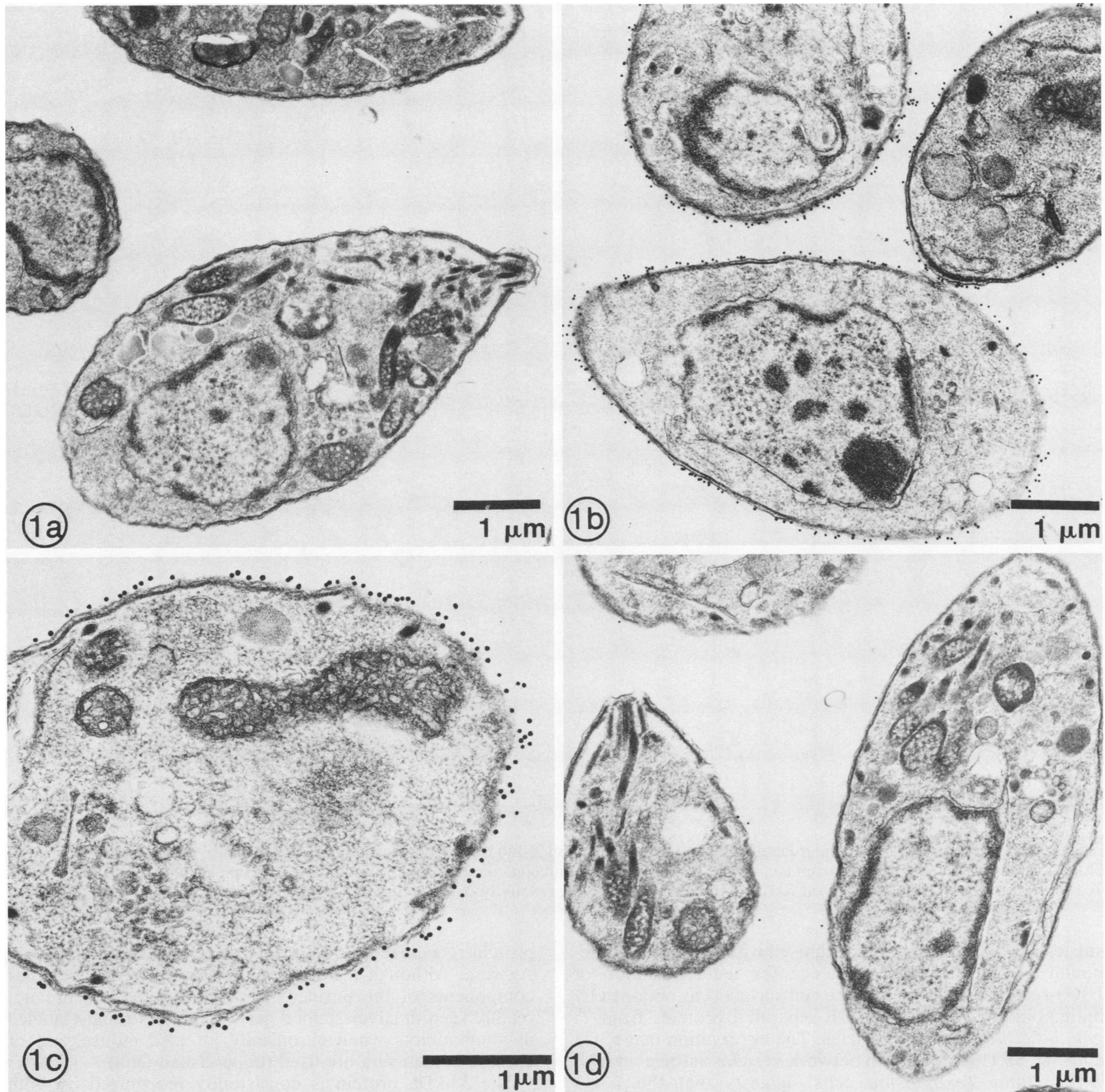

FIG. 1. Colloidal gold immunoelectron microscopy demonstrating reactivity with the surface of *Toxoplasma* cells. (a) Normal mouse sera; (b) chronically infected mouse sera; (c) monoclonal antibody 1E11 to the surface of the 30-kDa major surface protein; (2); (d) monoclonal antibody F3G3 to the 58-kDa cytoplasmic antigen.

tachyzoites. This result confirms previous observations that F3G3 recognizes a cytoplasmic antigen that is readily solubilized by hypotonic lysis (7). Although the epitope recognized by F3G3 is not normally exposed on the surface of intact *Toxoplasma* cells, it appears to be closely associated with the surface membrane. When *Toxoplasma* cells were fixed in the presence of Triton X-100, both polyclonal anti-F3G3-Ag sera and monoclonal antibodies F3G3 and 6-86-1E11 demonstrated specific immunoperoxidase labeling at what appeared to be the *Toxoplasma* cell surface. We interpret the requirement for Triton X-100 treatment as evidence that the reactive epitope is not normally exposed and must lie within or beneath the cell membrane. Similarly, Johnson et al. (4) have reported that monoclonal antibody FMC 20, which reacts with a cytoplasmic antigen, gives positive immunoperoxidase labeling at the cell surface following relatively strenuous fixation.

In addition to subsurface membrane labeling, specific immunoperoxidase labeling with F3G3 was detected in membrane vesicles that remained associated with extracellular *Toxoplasma* cells. Immunoperoxidase labeling of these vesicles by F3G3 was dependent on Triton X-100 treatment,

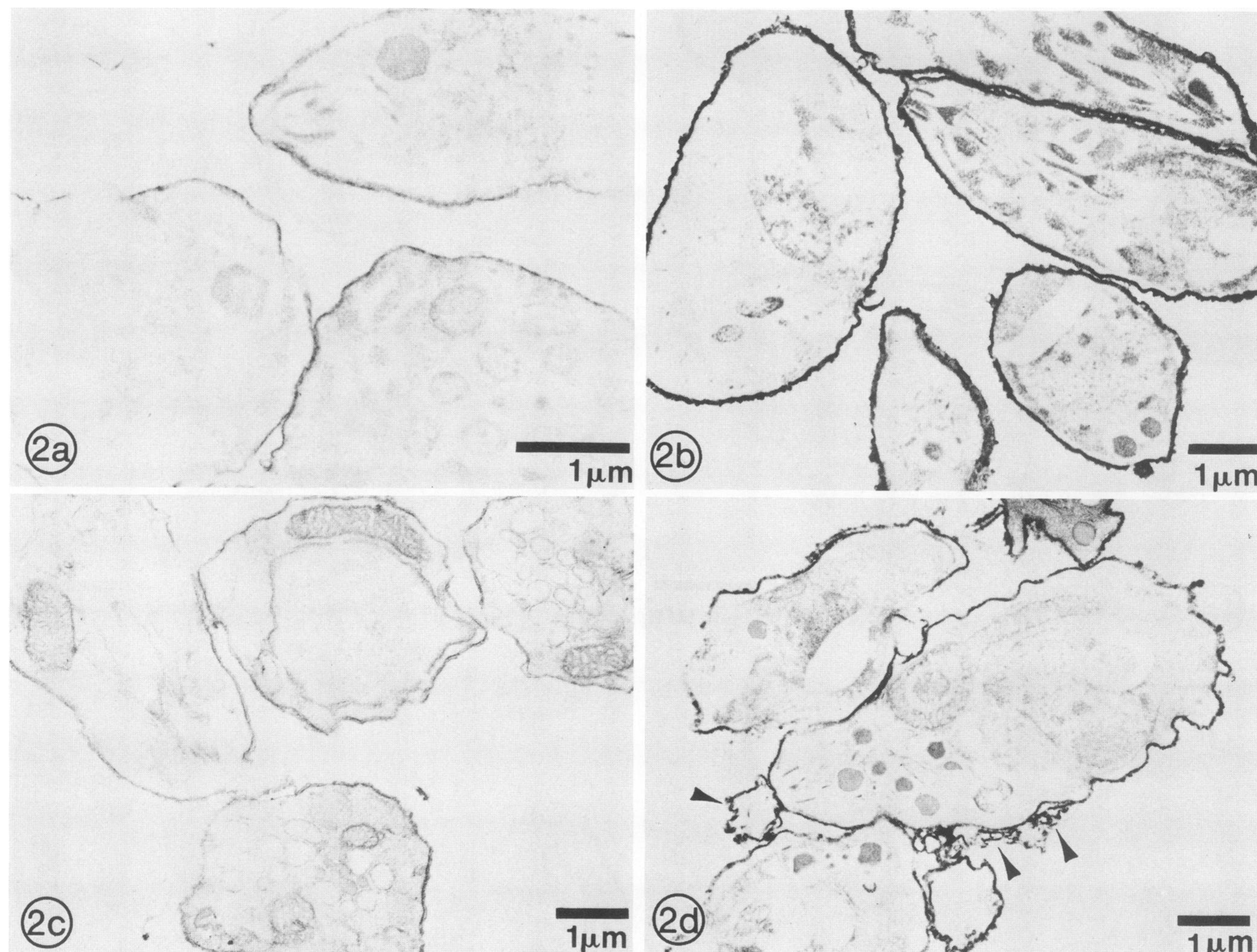

FIG. 2. Surface versus intracellular labeling distinguished by Triton X-100 treatment prior to immunoperoxidase electron microscopy. (a) Normal mouse sera on intact *Toxoplasma* cells; (b) chronically infected mouse sera on intact *Toxoplasma* cells; (c) F3G3 on intact *Toxoplasma* cells; (d) F3G3 on Triton X-100-extracted cells showing reactivity beneath the surface and in membrane vesicles (arrows).

suggesting that the epitope is enclosed within the membrane-bound vesicles. Further evidence for the association of F3G3-reactive proteins with the cell surface was obtained by immunoblotting of SDS-PAGE-separated proteins from *T. gondii*-derived network vesicles. The observation that F3G3 and 6-86-1E11 reacted with network vesicles associated with extracellular *Toxoplasma* cells suggests that the F3G3 epitope is also a component of the networks that are formed within host cells. We have demonstrated previously (9) a similar distribution of the major surface membrane protein of 30 kDa, which is found both on the *Toxoplasma* cell surface and within the intraphagosomal networks.

The pattern of proteins observed in silver-stained gels of the *T. gondii*-derived network vesicles is made up predominantly of 43-, 35-, 30-, and 20-kDa proteins (9), which is similar to the pattern of *Toxoplasma* proteins identified by surface iodination (2, 5). Indeed, it has been confirmed recently (L. D. Sibley, manuscript in preparation) that the major *T. gondii*-derived vesicle proteins react with monoclonal antibodies to known surface proteins of *Toxoplasma*. In the present study, analysis of *T. gondii*-derived vesicle proteins by immunoblotting demonstrated the presence of both a 28-kDa protein and lesser quantities of the 58-kDa protein recognized by 6-86-1E11 and polyclonal anti-F3G3-Ag sera. Although the 58- and 28-kDa antigens are minor components of the purified network vesicles and are not readily seen in silver-stained gels, they are strongly labeled in immunoblots when chronically infected mouse sera or chronic human sera are used (unpublished data).

The 58-kDa protein is consistently recognized by both 6-86-1E11 and polyclonal anti-F3G3-Ag sera in immunoblots of *Toxoplasma* lysate and corresponds to the apparent molecular weight of the F3G3 affinity-purified antigen analyzed by SDS-PAGE. The relationship between the 58- and the 28-kDa proteins is unclear, however, because we have not determined if these forms represent two proteins that share a common epitope or if they represent developmental or artifactual forms of the same protein. Further analysis of the processing of the 58-kD protein and its relationship to lower molecular weight proteins of *T. gondii* is presently under way.

In this report we have described the localization of 58- and 28-kDa proteins, which are recognized by monoclonal antibodies F3G3 and 6-86-1E11, that appear to be distributed beneath the cell surface membrane. The 58- and 28-kDa proteins also appear to be secreted from the cells enclosed

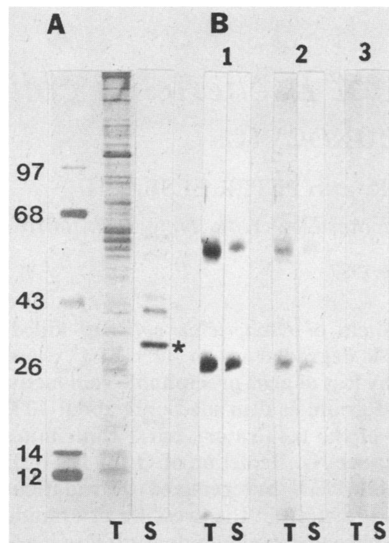

FIG. 3. Immunoblotting analysis of total *Toxoplasma* antigen (T; 20 μg per lane) and purified surface membrane vesicles (S; 5 μg per lane). Silver-stained (A) and ^{125}I-labeled protein A (B) autoradiographs. (B) Panel 1, polyclonal mouse sera raised against F3G3-purified antigen; panel 2, monoclonal antibody 6-86-1E11 raised against F3G3-purified antigen; panel 3, normal mouse sera. The asterisk denotes the major surface protein of 30 kDa in the silver-stained gel. Molecular weight markers are given in thousands.

within membrane vesicles that also contain the major *Toxoplasma* cell surface proteins. While not normally exposed extracellularly, there is evidence that the 58- and 28-kDa proteins can become exposed following mild disruption or some forms of chemical fixation (7, 8). Additionally, immunization with F3G3 affinity-purified protein induces antibodies that both fix complement on live *Toxoplasma* cells and agglutinate fixed cells (8). Moreover, passive transfer of F3G3 was sufficient to induce partial protection against lethal *Toxoplasma* challenge in mice (8). Although these observations are consistent with an extracellular or surface location of the F3G3-reactive epitope, we were not able to demonstrate significant surface labeling with F3G3 using freshly harvested *Toxoplasma* cells that were lightly fixed. Together, these observations suggest that the epitope recognized by F3G3 and 6-86-1E11 occurs on proteins that are located primarily beneath the surface membrane that can become exposed extracellularly after disruption of the outer cell membrane or perhaps during developmentally regulated release or secretion.

ACKNOWLEDGMENTS

This work was partially supported by Public Health Service grant AI 22585 from the National Institutes of Health.

LITERATURE CITED

1. **Araujo, F. G., and J. S. Remington.** 1984. Partially purified antigen preparations of *Toxoplasma gondii* protects against lethal infection in mice. Infect. Immun. **45**:122–126.
2. **Handman, E., J. W. Goding, and J. S. Remington.** 1980. Detection and characterization of membrane antigens of *Toxoplasma gondii*. J. Immunol. **124**:2578–2583.
3. **Handman, E., and J. S. Remington.** 1980. Serological and immunological characterization of monoclonal antibodies to *Toxoplasma gondii*. Immunology **40**:579–588.
4. **Johnson, A. M., W. D. Haynes, P. J. Lepard, P. J. McDonald, and S. H. Neoh.** 1983. Ultrastructural and biochemical studies on the immunohistochemistry of *Toxoplasma gondii* using monoclonal antibodies. Histochemistry **77**:209–215.
5. **Johnson, A. M., P. J. McDonald, and S. H. Neoh.** 1983. Monoclonal antibodies to *Toxoplasma gondii* cell membrane antigens protect mice from toxoplasmosis. J. Protozool. **30**:351–356.
6. **Laemmli, U. K.** 1979. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
7. **Naot, O., and J. S. Remington.** 1981. Use of enzyme linked immunosorbent assays (ELISA) for detection of monoclonal antibodies; experience with antigens of *Toxoplasma gondii*. J. Immunol. Methods **43**:333–339.
8. **Sharma, S. D., F. G. Araujo, and J. S. Remington.** 1984. *Toxoplasma* antigen isolated by affinity chromatography with monoclonal antibody protects mice against lethal infection with *Toxoplasma gondii*. J. Immunol. **133**:2818–2820.
9. **Sibley, L. D., J. L. Krahenbuhl, G. M. W. Adams, and E. Weidner.** 1986. *Toxoplasma* modifies macrophage phagosomes by secretion of a vesicular network rich in surface proteins. J. Cell Biol. **103**:867–874.
10. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.