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Electron Microscopic and Radioisotopic Studies on Cap Formation in *Toxoplasma gondii*

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Trophozoites of *Toxoplasma gondii* from mouse peritoneal exudate are capable of shedding antibodies with which they have been previously coated. The antibodies are first moved towards the anterior pole of the parasite, at which they form a "cap." From the pole they are shed into the environment in the form of antigen-antibody complexes. An internalization of the accumulated material has never been encountered. Most probably, the observed phenomenon reflects a mechanism by which the parasite evades the host's immune response.

Capping was originally described as polarization of immunoglobulin receptors of B lymphocytes under the influence of divalent antibodies directed against these receptors (15). However, it soon became evident that other mammalian cells (11, 14) and some protozoa (2, 3, 12) are also capable of accumulating various surface membrane components at one pole of the cell after being treated with antibodies or concanavalin A.

The procedure most frequently used to induce capping involves two steps. The cells are first coated with a layer of globulins that bind cell surface antigens and then are covered with a second layer of antiglobulin molecules that had been previously coupled with a marker, such as fluorescein isothiocyanate. If these cells are finally examined under ultraviolet light, any redistribution of their surface membrane antigens that has occurred is indicated by changes in the staining pattern. In cap-forming cells, an initially uniform fluorescence of the whole surface changes gradually to a patchy and then to a polar one.

When trophozoites of *Toxoplasma gondii* from mouse peritoneal exudate are subjected to the above procedure, some of them form fluorescent "caps" (4). These findings favored continuation of research on cap formation in *T. gondii* with the aid of other methods in the hope that they will throw some light on the nature of the phenomenon.

MATERIALS AND METHODS

Preparation of parasites. *T. gondii* on the RH strain were used throughout this study. The parasites were maintained in male CFW mice by syringe passage of the peritoneal exudate at 3- to 4-day intervals. The parasites in the peritoneal exudate were prepared for the studies by forcing the fluid through a surgical needle to release intracellular organisms and then diluting it with cold (0 to 4°C) TC medium (Difco) or Eagle TC minimal medium (Difco) supplemented with 10% heat-inactivated fetal calf serum (Colorado Serum Co., Denver, Colo.). The parasites were counted in a hemocytometer, and their number was adjusted to 10^7/ml in the diluting medium.

Antisera. High-titered human anti-*Toxoplasma* antisera were used throughout the experiments. All sera were heat inactivated at 56°C for 30 min, diluted with the medium, and tested against live parasites by the indirect fluorescent antibody test to find their optimum dilution inducing cap formation. This procedure was described in detail by Dzbeński and Zielińska (4). The percentage of cap-forming organisms was calculated separately for each of the antisera used. All observations aimed at establishing these data were made twice.

Labeling of anti-human IgG globulins. The following anti-human globulin preparations were used: (i) anti-human immunoglobulin G (IgG) globulin obtained from WHO International Reference Centre for Immunoglobulins, Lausanne, Switzerland; (ii) ammonium sulfate-precipitated IgG fraction of rabbit anti-human IgG obtained commercially from the Sera and Vaccine Institute, Warsaw. Both preparations were labeled with ^{125}I according to the method of McConahey and Dixon (9) and were then diluted with the medium to a concentration of 0.5 mg of protein per ml. The percentage of protein-bound iodine ranged in these preparations from 90 to 92, as calculated from the results of precipitation of the labeled samples with 20% trichloroacetic acid.

Anti-human IgG preparation (i) (above) was also labeled with peroxidase according to Nakane and Kawaoi (10). The enzyme preparation used was horseradish peroxidase type VI (Sigma Chemical Co., St. Louis, Mo.). Conjugated protein was separated from the unconjugated material by fractionation on a column of Sephadex G-100. The separated

conjugate was diluted with the medium to a final protein concentration of at least 0.5 mg of protein per ml immediately before use.

Autoradiography. Peritoneal exudate samples containing 10^7 trophozoites of *T. gondii* were incubated for 45 min at 0 to 4°C with the cap-inducing concentration of antisera. They were then washed three times with cold medium by centrifugation at 500 × *g*, reincubated with ^{125}I-labeled anti-human globulin for 30 min, and washed again. Some samples were subsequently placed on a water bath at 37°C, and the remaining ones were left at 0 to 4°C. After 2.5 h of incubation, all the samples were centrifuged in the cold, and the sediments were smeared on glass microscope slides, dried, and fixed with methanol. The slides were then covered with Ilford G5 liquid emulsion diluted with 9 volumes of distilled water and kept in light-tight boxes at 0 to 4°C for 7 to 21 days. After development, the slides were stained with Giemsa.

Electron microscopy. The parasites were initially treated as described for autoradiography except that peroxidase-labeled anti-human globulin was used instead of a radiolabeled one. After incubation with the antiglobulin and subsequent washings, the sediment of globulin-coated parasites was fixed for 5 min with cold 1.5% glutaraldehyde in phosphate-buffered saline at pH 7.2, washed in phosphate-buffered saline, and treated with 3,3'-diaminobenzidine containing 0.01% H_2O_2 (6). The parasites were then spun down, and the pellet was dehydrated by passing through a series of graded ethanols and embedded in an Epon 812 mixture. Sections were cut on a Porter-Blum ultramicrotome and mounted on copper grids. Some of them were stained with 1% uranyl acetate and lead citrate, and some were left unstained. All observations were carried out with a JEM 6C electron microscope.

Radioimmunoassay. The parasites were incubated with cap-inducing concentrations of antisera, washed with the medium, coated with ^{125}I-labeled anti-human globulin, and washed again as described for autoradiography. The washed sediments were subsequently checked for radioactivity on the scintillation counter USB-2 (POLON, Poland) and then supplemented with 1 ml of fresh medium and subjected to incubation at either 0 to 4 or 37°C. After 2.5 h, the parasites were spun down and the supernatants were pipetted off. The supernatants and sediments were checked on the counter to estimate percentage of loss of radioactivity that had passed from the parasites to the medium on incubation and then were treated in the following way. The sediments were reincubated with *Toxoplasma* antisera, washed, coated with fluorescein-labeled anti-human globulin, and examined under ultraviolet epi-illumination in a Leitz Ortholux fluorescent microscope. Radioactive supernatants, on the other hand, were mixed with an equal volume of polyethylene glycol 6000 (Carl Roth OHG, Karlsruhe, W. Germany) at a final concentration of 7.5% and left at 4°C for 18 h and then centrifuged at 1,000 × *g* for 20 min and checked on the counter for the presence of radioactive precipitates (1).

Measurements of anti-complementary activity. The supernatants collected after centrifugation of medium samples in which the globulin-coated parasites had been incubated for 2.5 h were tested for anti-complementary activity according to the procedure given by Shulman and Barker (13). The measurements were carried out twice with each of three *Toxoplasma* antisera used.

All these hemolytic assays were kindly performed by Irena Bragiel of the Institute of Haematology, Warsaw.

RESULTS

Trophozoites of *T. gondii* with a double layer of antibodies and then incubated at different temperatures differed in appearance according to the conditions of incubation.

The parasites incubated at 0 to 4°C showed an even distribution of labeled antibodies over the whole surface irrespective of whether fluorescence microscopy (Fig. 1), autoradiography (Fig. 2), or electron microscopy (Fig. 3) was used. In contrast to the above findings, some of the parasites that had been incubated at 37°C exhibited displacement of the attached label towards one pole of the cell (Fig. 4, 5, 6). Typical cap formation was found in some 5 to 20% of the individuals examined by the indirect fluorescent antibody test.

On autoradiographs, caps were made by fusion of individual silver grains into solid blocks that overshadowed one pole of the parasites (Fig. 5). Similar sized or larger blocks were scattered among the parasites throughout the microscope field (Fig. 7).

The parasites incubated at 0 to 4°C were mostly surrounded by separate grains, which never were observed to fuse at one pole (Fig. 2).

Electron microscopy of the parasites coated with peroxidase-labeled antibodies and then incubated at 0 to 4°C revealed the presence of a thin layer of electron-dense material lining the cells (Fig. 3). It was absent from the parasites still resident inside the macrophages. Most parasites incubated at 37°C presented a similar appearance. However, in some of them the label appeared to be pushed towards the anterior pole, where it formed a number of tufts of a hairlike structure (Fig. 8). These peroxidase-rich tufts measured 0.18 to 0.30 nm in diameter. They were also found in clusters among non-capped individuals. There were very few tufts in preparations of organisms incubated at 0 to 4°C. A number of the parasites incubated at 37°C showed vacuolization of the cytoplasm (Fig. 9).

The results of radioimmunoassays performed with the aid of three different *Toxoplasma* antisera and two iodine-labeled antiglobulins indicated that the amount of radioactivity passing from the labeled parasites to the medium on

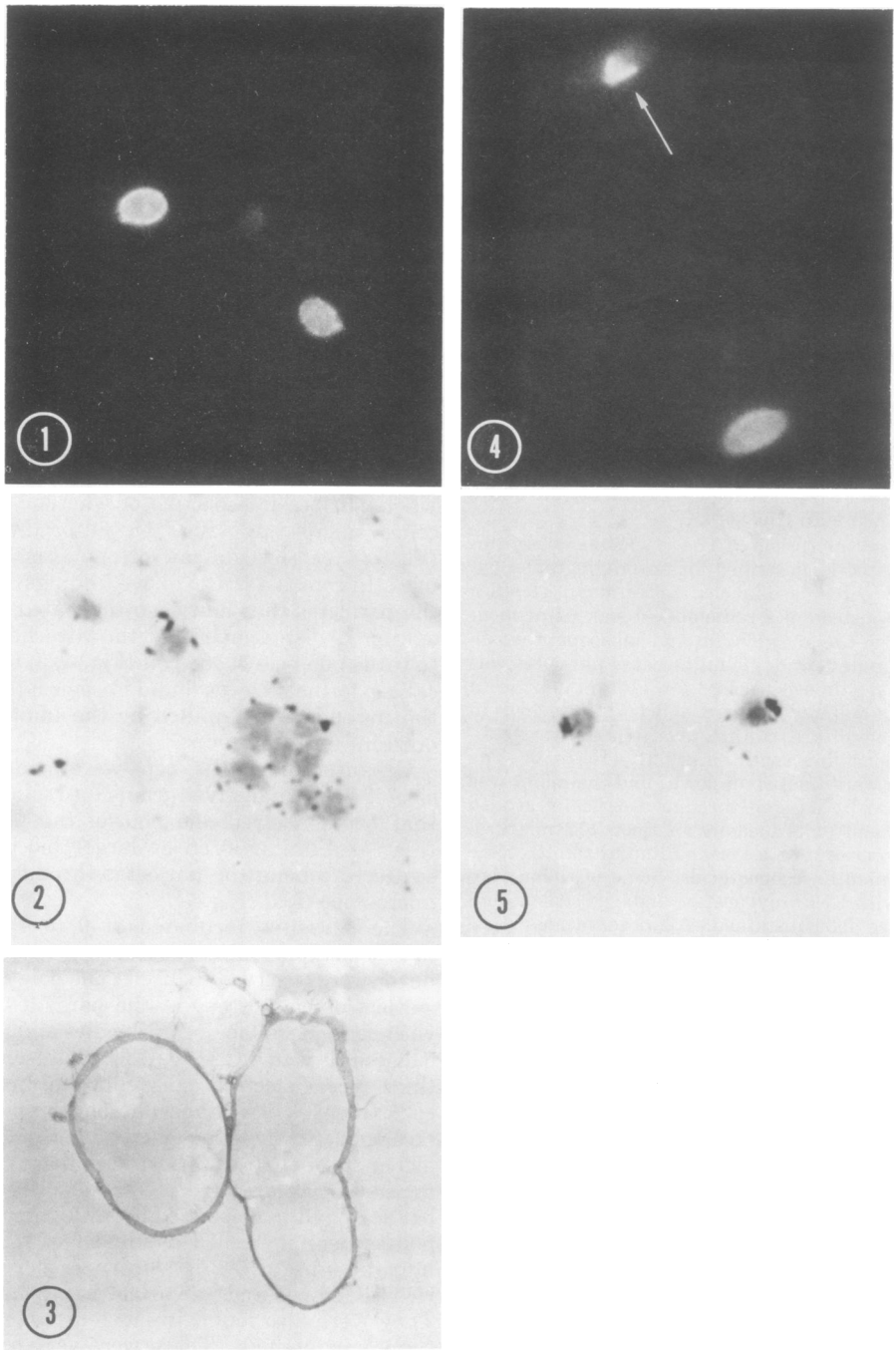


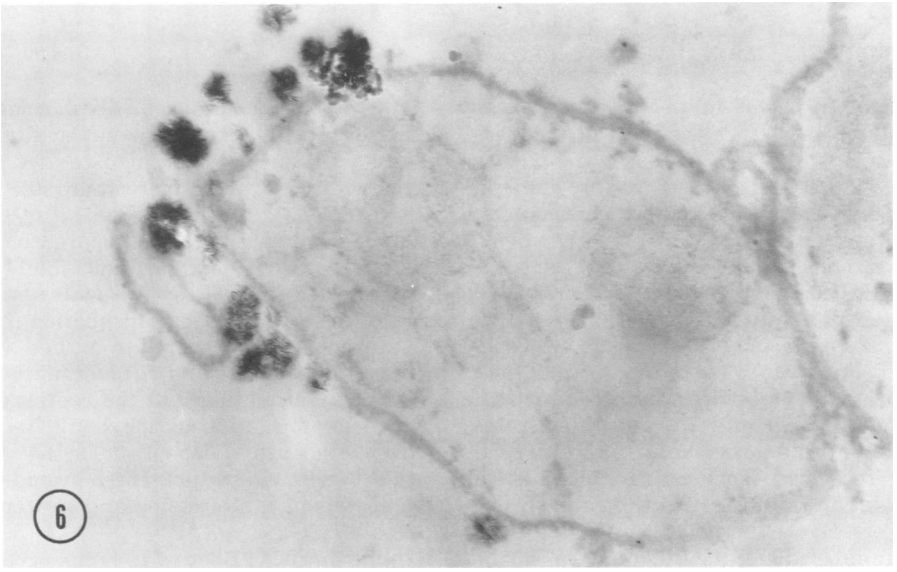
FIG. 1. *Fluorescence pattern of living trophozoites of T. gondii which were coated with two successive layers of human antibodies and fluorescein-labeled anti-human globulin and then left at 0 to 4°C. ×2,090.*

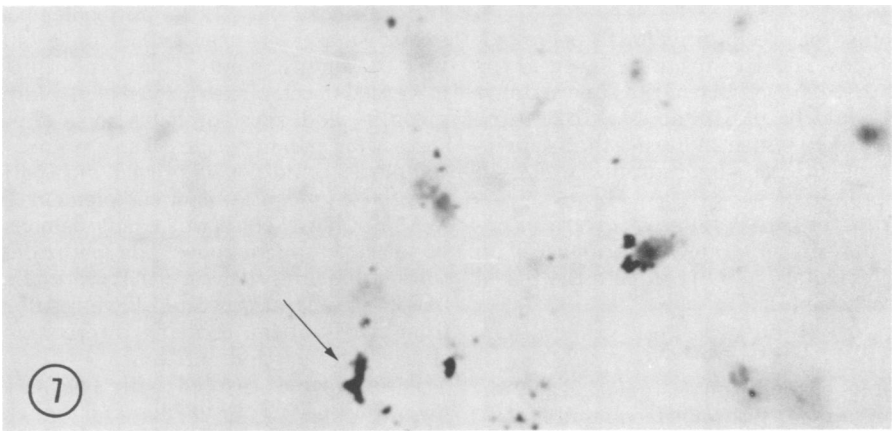
FIG. 2. *Autoradiograph of T. gondii trophozoites coated with human antibodies and ^{125}I-labeled antiglobulin at 0 to 4°C. ×3,560.*

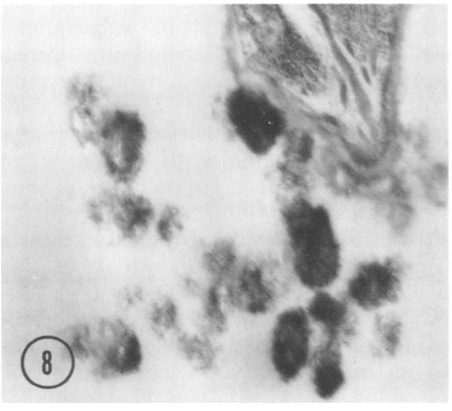
FIG. 3. *Electron micrograph of a group of trophozoites of T. gondii coated with peroxidase-labeled antibodies and then incubated at 0 to 4°C. ×9,000.*

FIG. 4. *Fluorescence pattern of living trophozoites of T. gondii coated with a double layer of antibodies and then incubated at 37°C. Arrow points to the individual with a fluorescent cap. ×3,290.*

FIG. 5. *Autoradiograph of T. gondii trophozoites coated with antibodies as described for Fig. 2 but incubated at 37°C. Note the presence of silver grain caps over both individuals. ×3,560.*







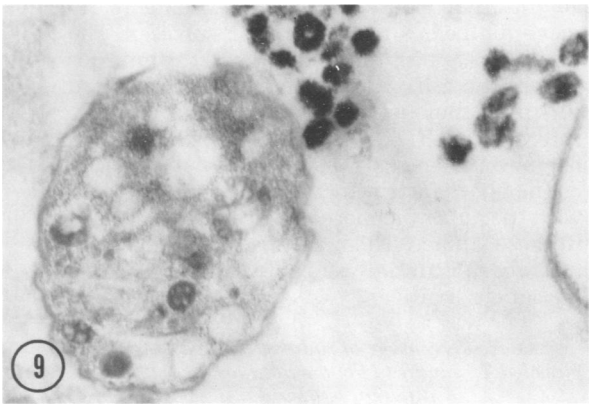


FIG. 6. *Electron micrograph of a trophozoite of T. gondii coated with antibodies and peroxidase-labeled antiglobulin and incubated at 37°C. Note the presence of peroxidase-rich tufts forming a cap at one pole of this cell.* ×26,000.

FIG. 7. *Autoradiograph of trophozoites of T. gondii treated like the parasites shown in Fig. 5. Arrow points to the blocks of grains that are frequent in such preparations.* ×3,560.

FIG. 8. *Electron micrograph of a trophozoite of T. gondii showing anterior pole of the parasite covered with antibodies and incubated at 37°C. Note the number of peroxidase-rich tufts in the nearest vicinity of parasite's pole. Uranyl acetate-lead citrate staining.* ×25,300.

FIG. 9. *Electron micrograph of an antibody-coated trophozoite of T. gondii after incubation at 37°C. Note vacuolization of parasite cytoplasm. Uranyl acetate-lead citrate staining.* ×16,500.

incubation at 37°C was 4.5 to 11.7 times larger than that released from the cells at 0 to 4°C (Table 1).

Although some parasites inevitably suffered death during the course of the experiments, most of them remained alive and virulent, since 10^6 toxoplasmas recovered from these experiments killed the mice within 4 to 5 days of intraperitoneal administration.

The capability of these parasites to bind specific antibodies was also retained, and they showed bright fluorescence when treated with human *Toxoplasma* antisera and fluorescein-conjugated anti-human globulin. However, the parasites recovered from radioimmunoassays conducted at 37°C fluoresced more intensively. The fluorescence of the parasites treated with fluorescein-labeled antiglobulin only was negligible.

The method of precipitation with polyethylene glycol gave negative results. However, virtually all the radioactivity of the supernatants examined could be precipitated with 20% trichloroacetic acid, which indicated that radioiodine present in the supernatants was still bound to proteins.

The results of hemolytic assays were inconclusive as they demonstrated the presence of an anti-complementary activity in both the tested and control samples.

DISCUSSION

The results of the present studies clearly indicated the capability of some trophozoites of *T. gondii* from mouse peritoneal exudate to remove antibodies bound to their surfaces. The antibodies are first moved towards the anterior pole of the cell, where they form a cap. From this pole, they are shed into the environment. Internalization of the accumulated material has never been observed.

The above facts were unequivocally revealed with the aid of the morphological methods used in these studies. The results of the radioimmunoassay substantially supported these morphological observations.

TABLE 1. *Percentage of radioactivity passing from labeled T. gondii to the medium on incubation at different temperatures*

Sera	Source of ^{125}I-labeled antiglobulin	% of radioactivity at temp of:	
		37°C	0 to 4°C
H	Sera and Vaccine Institute	41.4	6.6
H_2	Sera and Vaccine Institute	29.3	2.5
H_3	WHO	24.1	5.3

Capping could be induced only in some organisms. Under the conditions of the studies, it was usually encountered in 5 to 20% of the parasites but never in more than 50% of the examined cells.

Parasite capping does not apparently reflect any degenerative changes that might have been expected in cells covered with antibodies and subjected to prolonged incubation at 37°C. Most toxoplasmas recovered from these experiments remained alive and virulent, as tested by animal subinoculation. On the contrary, it is obvious that formation of caps should be considered a metabolically dependent, active process since it was inevitably prevented by addition of some metabolic inhibitors (4) and low temperatures (0 to 4°C).

Unlike antibodies bound to lymphocytes (15, 16) or tumor cells (7), the antibodies bound to toxoplasmas were not endocytosed but shed into the environment. These antibodies were discarded in complexes formed of globulin, antiglobulin, and, most probably, also of parasite surface antigen molecules. The presence of such complexes could not be proved with polyethylene glycol precipitation or complement fixation methods; however, it was easily demonstrated by electron microscopy. The peroxidase-rich tufts of hairlike structure that were abundant in some preparations could have resulted only from cross-linkage of many interacting molecules of antigen and antibodies.

The parasites covered with two successive layers of specific antibodies and radiolabeled antiglobulin discarded them both under capping conditions and could be subsequently stained with fluorescein-labeled anti-human globulin. However, fluorescence of the parasites treated in the manner described above was much weaker in comparison with that of the parasites incubated with human *Toxoplasma* antibodies, once again prior to being exposed to fluorescein-labeled antiglobulin.

Results of this experiment indicated that labeled antiglobulins are shed by parasites together with anti-parasite antibodies.

Does the surface antigen of toxoplasmas share the fate of these antibodies, or is it retained, entirely or partly, on the cell to enable binding of antibodies at the second exposure?

We think that the antigen is discarded by parasites together with its corresponding antibody, although direct evidence for this has not been obtained. The parasites capable of discarding their surface antigen are also capable of synthesizing it de novo. Frequent vacuolization of parasites under capping conditions may actually reflect the process of antigen synthesis. A vacuolated parasite is very much like a de-

veloping oocyst of *T. gondii* with its wall-forming bodies (5). The vacuoles would form a new layer of surface antigen in a manner similar to that in which wall-forming bodies make an oocyst wall.

In conclusion, we believe that capping in the trophozoites of *T. gondii* reflects a mechanism by which the parasite evades the host's immune response. *T. gondii*, which is the obligate intracellular parasite, developed principally such mechanisms of defense as would enable it to survive within the invaded cells. According to Jones and Hirsch (8), these mechanisms involve prevention of the delivery of lysosomal contents to phagocytic vacuoles containing living toxoplasmas. Within the invaded cells the parasites are fully protected against host antibodies. However, every parasite-harboring cell must finally burst open, and then the toxoplasmas will be in contact with host antibodies before even having a chance to invade new cells. Under these circumstances, they are forced to use other means of escape, which is sometimes achieved by cap formation.

The above idea was developed on the grounds of experiments carried out in vitro on toxoplasma that were covered with a double layer of antibodies. We do not know whether the parasites are capable of cap formation under the influence of anti-*Toxoplasma* antibodies alone, a situation more likely to occur in vivo.

Can we, in that case, use the results of our double-labeling experiments as an argument for the idea that capping is one of toxoplasma's escape mechanisms?

We think we can, because double labeling of parasites may also occur in vivo, namely, under the influence of anti-*Toxoplasma* antibodies and rheumatoid factor, which is quite frequently present in the sera of subjects infected with this protozoan.

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