Parasitol Res (1990) 76:559–562

Parasitology Research

Exocytosis of *Toxoplasma gondii* dense granules into the parasitophorous vacuole after host cell invasion

Marie Anne Leriche and Jean François Dubremetz

Unité 42 INSERM, 369, Rue Jules Guesde, 59650 Villeneuve d'Ascq, France

Accepted March 10, 1990

Abstract. Tachyzoites of *Toxoplasma gondii* have been shown to exocytose the contents of dense granules into the parasitophorous vacuole after host cell invasion. A monoclonal antibody specific for a 27-kDa protein was used to locate the dense granules by immunoelectron microscopy. The same antibody also reacted with the tubular network found in the parasitophorous vacuole, which confirmed that the dense granules were exocytosed by tachyzoites.

Dense granules of sporozoan zoites have received little attention because they have often been confused with cross sections of rhoptries, which are organelles belonging to the zoite apical complex. These granules have mostly been studied in the genus *Sarcocystis*, from which they have been isolated by subcellular fractionation and characterized (Dubremetz and Dissous 1980) and in which they were subsequently shown to be released into the secondary parasitophorous vacuole after host cell invasion (Entzeroth 1984; Entzeroth et al. 1986). Electron-dense granules are also present in other sporozoan zoites, especially in *Toxoplasma gondii* (Vivier and Petitprez 1972), but a possible analogy with the phenomenon observed in *Sarcocystis* has not yet been described. We obtained electron micrographs of intracellular *Toxoplasma* tachyzoites that suggest the release of the contents of dense granules into the parasitophorous vacuole. By using a monoclonal antibody, we identified a 27-kDa protein in dense granules that is also present in the tubular network in the parasitophorous vacuole of *T. gondii* – infected cells. These results are reported herein.

Materials and methods

Parasites

All experiments were performed with tachyzoites of the RH strain of *T. gondii*. These were routinely maintained by successive passage in the peritoneal cavity of Swiss mice. For all experiments described, tachyzoites were produced in monolayers of HeLa cells, which were maintained in Dulbecco's minimal essential medium supplemented with 5% fetal calf serum. Parasites were purified from infected monolayers by glass-wool filtration.

Reprint requests to: M.A. Leriche

Monoclonal antibody production

Monoclonal antibodies (McAbs) were obtained by fusion of SP2/0 myeloma cells with splenocytes of BALB/c mice that had been immunized with a rhoptry-dense-granule fraction of *T. gondii* (Leriche and Dubremetz, in preparation). The cell fusion was carried out according to Galfre et al. (1977). Screening of hybridomas was done by immunofluorescence assay and Western blotting of tachyzoite lysates (see below). Positive hybridomas were cloned by limiting dilution. Mass production of McAbs was done by injection of hybridoma cells into pristane-primed BALB/c mice.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

This procedure (SDS-PAGE) was performed according to Laemmli (1970), using 12% (w/v) acrylamide separating gels. Molecular weight standards (Pharmacia, LMW kit) were used for estimating the apparent molecular weight of the proteins analyzed.

Western blotting

Purified tachyzoites were analyzed by SDS-PAGE under non-reducing or reducing conditions (10^7 zoites/1-cm-wide slot) and electrophoretically transferred to nitrocellulose (200 mA, 1 h; Towbin et al. 1978). The nitrocellulose sheet was then saturated for 30 min in 5% nonfat dry milk in buffer [140 m*M* NaCl and 0.5% (v/v) Tween 20 in 15 m*M* TRIS-HCl (pH 8) (TNT)]. Strips were then cut and incubated with the McAb solutions (pure hybridoma culture fluid or mouse ascitic fluid diluted 1:200 in TNT) for 1 h at 37° C. After being washed, the strips were incubated in alkaline phosphatase-conjugated antimouse IgG (Promega) diluted in TNT and revealed with nitroblue tetrazolium.

Immunofluorescence assay

For hybridoma screening, purified RH tachyzoites were washed three times with phosphate-buffered saline [PBS: 150 m*M* NaCl,

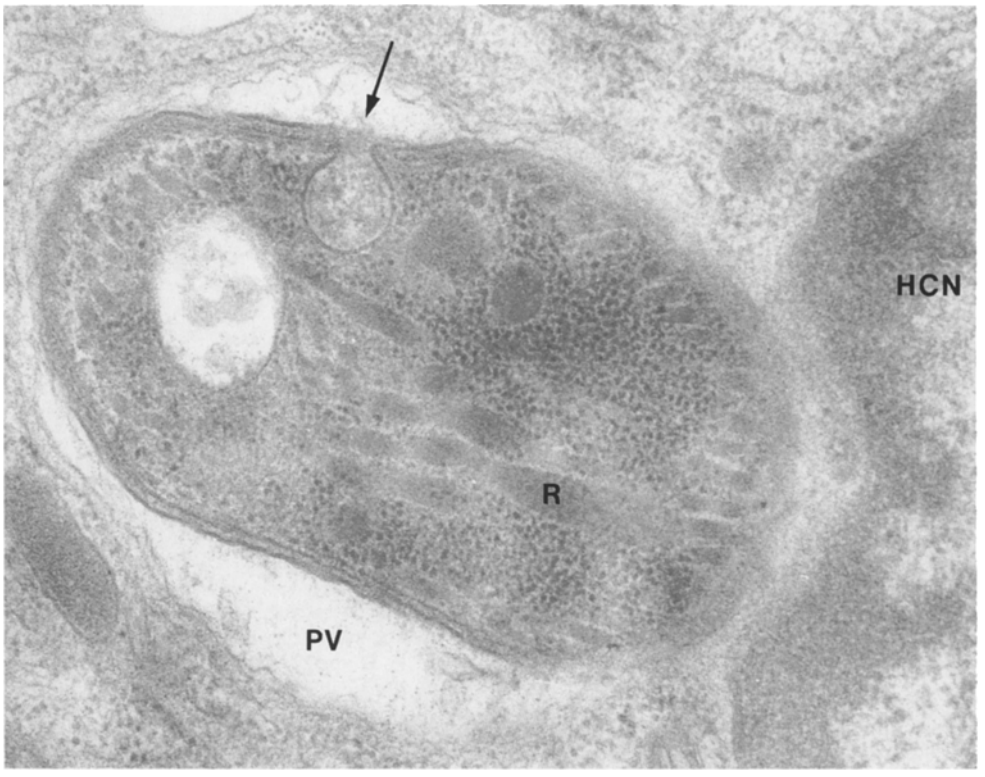

Fig. 1. Newly invaded *T. gondii* tachyzoite in which a vesicle open at the zoite surface communicates with the vacuolar space, suggesting exocytosis of its contents (*arrow*). *HCN*, host cell nucleus; *PV*, parasitophorous vacuole; *R*, rhoptry. ×58,500

50 m*M* phosphate buffer (pH 7.4)] and dried on standard immunofluorescence assay (IFA) slides, which were stored at −20° C. IFA was carried out at 37° C after a 10-min fixation in cold acetone. Slides were incubated with McAbs (hybridoma culture medium), washed, and incubated in fluorescein-conjugated rabbit antimouse IgG antibodies.

Electron microscopy

For standard electron microscopy, a monolayer of HeLa cells was incubated for 15 min at 37° C with tachyzoites that had been sedimented on the cells for 15 min at 4° C to synchronize invasion. It was then fixed for 1 h in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.3), washed in the same buffer, and postfixed for 1 h in 1% osmium tetroxide in the same buffer. The monolayer was then dehydrated in ethanol and embedded in Epon. Embedded cells were sectioned with a diamond knife and contrasted with uranyl acetate and lead hydroxide.

For immunoelectron microscopy, ascitic cells from a mouse infected 60 h earlier with 1.2×10^8 tachyzoites and 10^8 TG 180 sarcoma cells were washed twice in PBS and fixed for 30 min in 2% paraformaldehyde/0.1% glutaraldehyde in PBS. They were washed in PBS, dehydrated in ethanol, and embedded in Lowicryl K4M at −30° C. Thin sections were collected on carbon-coated nickel grids; they were floated for 30 min on 1% ovalbumin in PBS (PBSO) and were transferred on McAb T5 2B4 (undiluted culture medium) for 1 h. After being washed with PBS, the grids were floated on rabbit antimouse IgG serum (Tago) diluted 1:50 in PBSO for 1 h, then washed and transferred for 1 h on 8 nm protein A-gold that had been diluted 1:25 in PBSO (OD 525:0.05). Thin sections were stained with 3% uranyl acetate in water and observed with a Hitachi H600 electron microscope.

Results

Newly invaded *T. gondii* tachyzoites were found within a membrane-bounded parasitophorous vacuole that was usually surrounded by a layer of endoplasmic reticulum

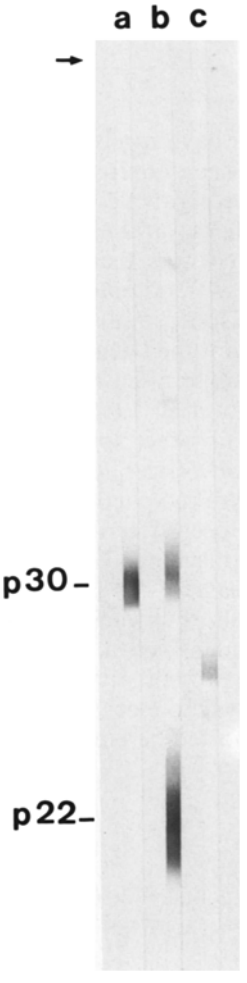

Fig. 2. Western blotting of *T. gondii* tachyzoites probed with McAb T5 2B4 (*c*). For calibration, the same blot was probed with McAb specific for surface antigens (*a*, P30; *b*, P22)

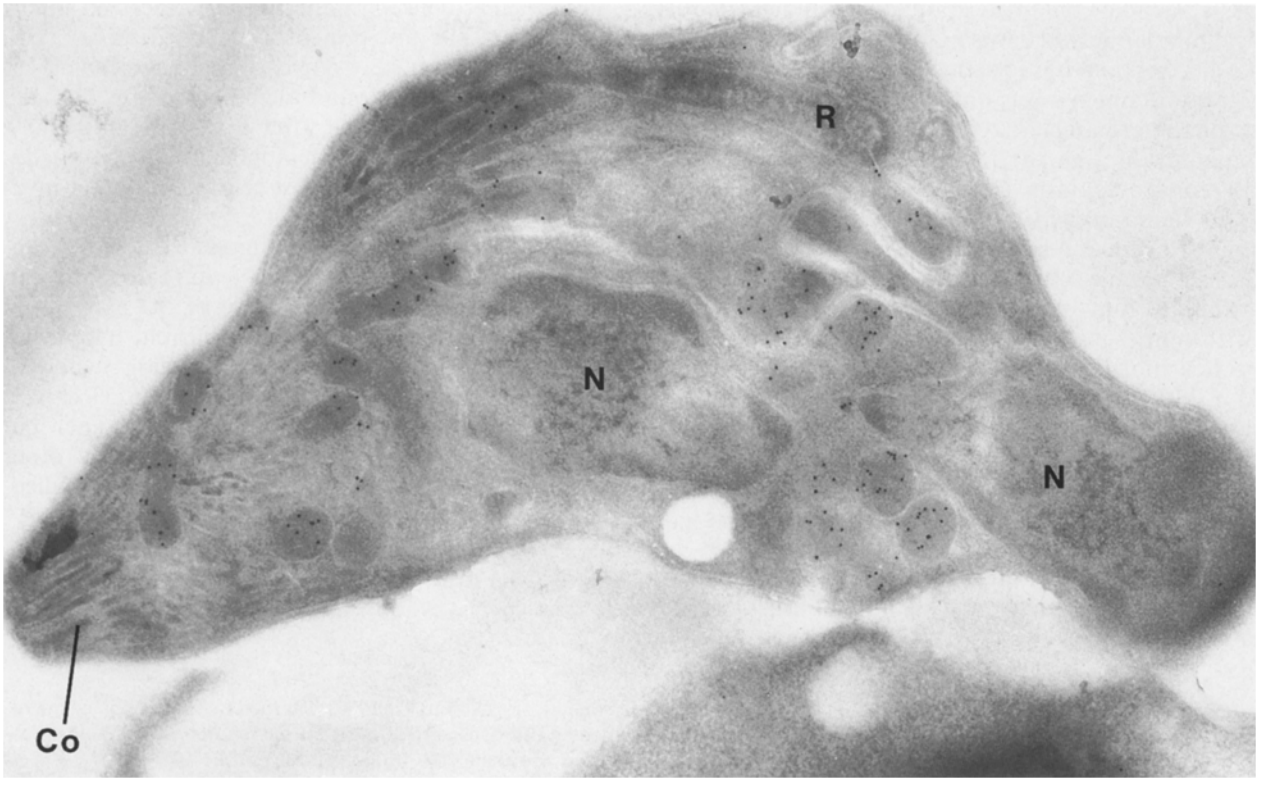

Fig. 3. Immunogold localisation of McAb T5 2B4 on a Lowicryl section of a *T. gondii* tachyzoite (at the end of endodyogeny): dense granules are specifically labeled. *Co*, conoid; *N*, nucleus. ×28,000

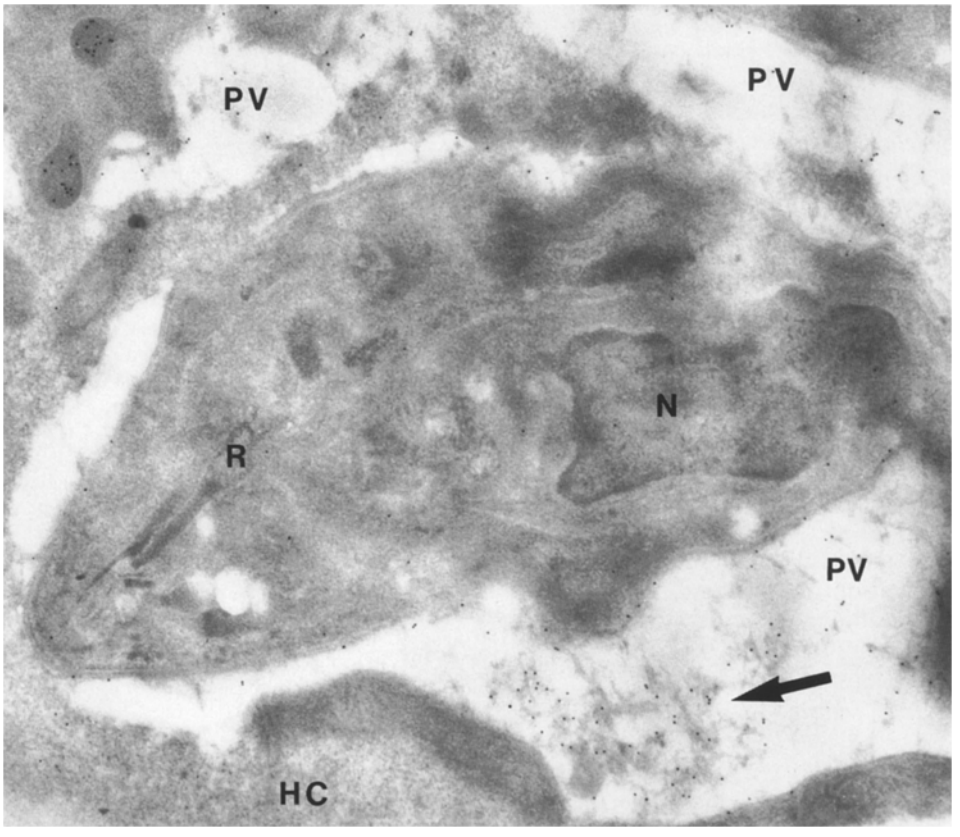

Fig. 4. Immunogold localisation of McAb T5 2B4 on a Lowicryl section of an intracellular *T. gondii* tachyzoite: most of the label is found on the tubular network (*arrow*) located in the parasitophorous vacuole. *HC*, host cell. ×28,000

(Fig. 1). The vacuole contained granulo-vesicular material; typical tubular network was not found in these early stages. An electron micrograph suggesting the exocytosis of dense-granule contents was obtained; it showed a circular invagination (diameter, 200 nm) of the plasmalemma of the zoite through the inner membrane complex within the zoite cytoplasm (Fig. 1). Granulo-vesicular material was found in the vesicle and in the vacuole.

During the production of McAbs against a *T. gondii* rhoptry-dense-granule subcellular fraction, IFA revealed one hybridoma that was secreting antibodies that reacted with numerous dots scattered in the cytoplasm of tachyzoites (not shown). This hybridoma (T5 2B4) identified a 27-kDa protein in Western blots of tachyzoite lysates under both reducing and nonreducing conditions (Fig. 2). On Lowicryl ultrathin sections of tachyzoites, this McAb reacted exclusively with dense granules (Fig. 3) and no label was found on other organelles. When applied on *T. gondii* -infected cells, the label was also found in the parasitophorous vacuole, mostly located on the tubules of the network (Fig. 4); no label was found on uninfected cells.

Discussion

The present study demonstrates the exocytosis of *T. gondii* tachyzoite dense-granule contents into the parasitophorous vacuole soon after invasion. This supports the suggestion of Sibley and Krahenbuhl (1988) that they found components of dense granules in the contents of the parasitophorous vacuole. Moreover, the present study resulted in the observation of dense-granule exocytosis, the identification of a 27-kDa protein distinct from the 32-kDa molecule described by Sibley, and the use of post-embedding immunoelectron microscopy, which leads to more accuracy in immunodetection.

The observation of exocytosing dense granules is quite similar to that reported for *Sarcocystis* (Entzeroth 1984). One might wonder whether this phenomenon could be distinguished from endocytosis through micropores, which has also been described in Coccidia (Scholtyseck and Mehlhorn 1970); the absence of thickening of the inner-membrane complex around the opening of the dense granule could be a morphological criterion for differentiating both phenomena.

The intravacuolar tubular network was described by Sibley et al. (1986) as being shed by the zoite into the vacuole (or extracellularly under certain ionic conditions). These authors suggested a major contribution to this network by surface antigens and, more recently, by dense-granule components (Sibley and Krahenbuhl 1988). Nichols et al. (1983) observed identical tubules within organelles that they identified as rhoptries during *T. gondii* invasion. The origin of the tubular network might thus be complex, and further studies are needed to identify the contribution of different organelles to this structure.

The 27-kDa dense-granule protein described in the present report has not previously been identified with McAbs. However, it may correspond to the major 25-kDa molecule that was found to be released by *T. gondii* tachyzoites in the host cell after invasion (Dubremetz et al. 1982) by the use of metabolically labeled tachyzoites and the disruption of host cells 30 min after invasion.

Taken together, these results suggest that dense-granule exocytosis might be a common feature in sporozoan zoite-host cell interactions. The role of the exocytosed components is unknown, although a current hypothesis is that they would play a part in avoiding fusion of lysosomes with the parasitophorous vacuole. However, the parasitophorous vacuole is not a phagosome, and the interaction between that structure and the other compartments of the host cell has yet to be studied. Dense granules may play a role in this interaction.

Acknowledgements. The authors are indebted to C. Ansel, I. Briche, A. Loyens, and M. Mortuaire for their helpful assistance. This work was supported by INSERM.

References

Dubremetz JF, Dissous C (1980) Characteristic proteins of micronemes and dense granules from *Sarcocystis tenella* zoites (Protozoa, Coccidia). Mol Biochem Parasitol 1:279–289

Dubremetz JF, Dissous C, Ferreira E (1982) *Toxoplasma gondii*: liberation d'une proteine 25 kD associée à l'invasion de la cellule hôte (abstract). J Protozool 29:303 [A]

Entzeroth R (1984) Electron microscope study of host parasite interaction of *Sarcocystis muris* (Protozoa, Coccidia) in tissue culture and in vivo. Z Parazitenkd 70:131–134

Entzeroth R, Dubremetz JF, Hodick D, Ferreira E (1986) Immunoelectron microscopic demonstration of exocytosis of dense granule contents into the secondary parasitophorous vacuole of *Sarcocystis muris* (Protozoa, Apicomplexa). Eur J Cell Biol 41:182–188

Galfre G, Howe SC, Milstein C, Butcher GW, Howard JC (1977) Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature 266:550–552

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Nichols BA, Chiappino ML, O'Connor GR (1983) Secretion from the rhoptries of *Toxoplasma gondii* during host cell invasion. J Ultrastruct Res 83:85–98

Scholtyseck E, Mehlhorn H (1970) Ultrastructural study of characteristic organelles (paired organelles, micronemes, micropores) of Sporozoa and related organisms. Z Parasitenkd 34:97–127

Sibley LD, Krahenbuhl JL (1988) Modification of host cell phagosomes by *Toxoplasma gondii* involves redistribution of surface proteins and secretion of a 32 kDa protein. Eur J Cell Biol 47:81–87

Sibley LD, Krahenbuhl JL, Adams GMW, Weidner E (1986) Toxoplasma modifies macrophage phagosomes by secretion of a vesicular network rich in surface proteins. J Cell Biol 103:867–874

Towbin H, Stahelin T, Gordon J (1978) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

Vivier E, Petitprez A (1972) Données ultrastructurales complémentaires morphologiques et cytochimiques sur *Toxoplasma gondii*. Protistologica 8:199–221

Note added in proof

After submission of this paper, we became aware of the work by M.F. Cesbron-Delauw et al. (Proc Natl Acad Sci USA 86:7537–7541, 1989) and exchanged antibodies with these authors. Western blot comparison showed comigration of the antigens and suggested that the 23 kDa protein cloned by these authors and the 27 kDa described here are probably the same molecule.