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Role of Antibody and Complement in the Control of *Encephalitozoon cuniculi* Infections by Rabbit Macrophages

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The capacity of mononuclear peritoneal macrophages to phagocytose *Encephalitozoon cuniculi* was tested in vitro. Normal rabbit serum or cell culture medium had little effect on the rate of removal of organisms by rabbit peritoneal macrophages. Treatment with immune rabbit serum or immune rabbit immunoglobulin G significantly ($P < 0.001$) increased phagocytosis of *E. cuniculi*. Guinea pig complement was found to significantly ($P < 0.001$) enhance the phagocytosis of antibody-treated *E. cuniculi*. With few exceptions, induced (peritoneal exudate) macrophages were no more effective than unstimulated (resident) macrophages in the phagocytosis of *E. cuniculi*. Secondary lysosomes labeled with ferritin were seen fusing with phagosomes containing immune rabbit serum-treated parasites. Phagosome-lysosome fusion was not observed when parasites were treated with either normal rabbit serum or culture medium. The results of the present study suggest a role for antibody enhancement of phagocytosis and intracellular killing as a mechanism of resistance to encephalitozoonosis in rabbits.

Encephalitozoon cuniculi is an obligate intracellular protozoan parasite that develops within a membrane-lined vacuole within the cytoplasm of mammalian cells (24). The infection is initiated either by intracytoplasmic insertion of the parasite's genome via the sporoplasm on the end of an extruded polar filament or by endocytosis of sporoplasm or spores (24). Once inside the host cytoplasm the parasite divides by binary fission to produce multiple schizonts. Schizonts are capable of multiple divisions but ultimately enter a sequence of division and maturation culminating in spores (24). The entire process is completed in the membrane-lined cytoplasmic vacuole and ends when the cell is so distended with parasites that it ruptures. At no time during the process is there fusion of the vacuole, known as the parasitophorous vacuole, with lysosomes, despite the fact that the parasites infect peritoneal macrophages rich in lysosomes (38).

The mechanism(s) by which fusion between lysosomes and parasitophorous vacuoles is prevented is unknown. Other intracellular parasites can escape intracellular killing by a variety of methods. For example, vaccinia virus (8), *Babesia* (30), and trypanosomes (21) lyse the macrophage phagosome and escape into the cytoplasmic milieu where they replicate. Other parasites, e.g., *Chlamydia* (12) and *Toxoplasma* (17), avoid intracellular hydrolysis by preventing phagosome-lysosome fusion. Endocytosed *Leishmania* remain within macrophage phagosomes which then fuse with lysosomes (7). These parasites are impervious to lysosomal hydrolases and reside within the lysosome as part of their life cycle.

Introduction of *E. cuniculi* to cell cultures in vitro results in heavy infections in which most cells are parasitized and the culture is destroyed. Infected animals, on the other hand, have a very limited disease characterized in the rabbit by multifocal granulomatous encephalitis and lymphocytic interstitial nephritis (18, 20, 22, 25, 28, 31). In some cerebral lesions, numerous parasites can be seen in a central area of caseation necrosis surrounded by a broad zone of apparently uninfected macrophages (33). Parasitized cells occur away from the sites of inflammation and are characterized by large parasitophorous vacuoles filled with parasites. The disease rarely results in clinical signs in rabbits, and the lesions are always focal and scattered (33). Even in the dog, in which the disease is clinically and anatomically much more severe, the lesions are focal and confined to a few organs and tissues (3, 26, 27). Infected animals thus limit the dissemination of the parasite, but the mechanism(s) by which this is accomplished is unknown.

Infected animals produce antibody detectable by several serological methods (39; J. Chalupsky, Protozoology **18** (Suppl.): 47, 1971), and there is also presumptive evidence for sensitized T-lymphocytes (23). Hyperimmune serum with or without complement is not capable of mediating parasite destruction as determined in a simple neutralization test in rabbit fibroblast mono-

layers (unpublished data). It seems unlikely that sensitized T-lymphocytes alone can kill spores directly because of the extremely thick spore wall. Although spores cannot be lysed by antibody and complement, it might be that adhesion of antibody or complement or both to the surface of spores or macrophages might convert the macrophage from a suitable host cell to one capable of destroying the parasite. This suggestion also is compatible with observations in vivo in which animals with antibody exhibit a vigorous macrophage reaction to parasites free in tissue and the macrophages in these lesions do not contain parasitophorous vacuoles.

Since *E. cuniculi* can evade intracellular killing and successfully reside within the macrophage, we wished to determine whether the macrophage could be converted from a susceptible host cell to an effector cell capable of killing this parasite and thereby functioning as a mediator of resistance to *E. cuniculi* infections.

MATERIALS AND METHODS

IFAT screening of rabbit serum. Rabbit serum was examined by the indirect fluorescent-antibody test (IFAT) (42) with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (IgG) (lot no. S358; Miles Laboratories, Elkhart, Ind.). Undiluted serum samples failing to demonstrate immunofluorescence were considered negative and were used as normal rabbit serum controls or as the source of normal rabbit IgG. A serum sample with an IFAT titer of 1:1,024 was designated *E. cuniculi* immune rabbit serum.

IgG preparation. IgG was separated from normal or immune rabbit serum by chromatography in diethylaminoethyl-cellulose (Sigma Chemical Co., St. Louis, Mo.) (11). IgG samples were tested for antibodies against *E. cuniculi* with IFAT. The immune IgG titer was 1:512 (total protein 2.2 mg/ml [19]). Normal rabbit IgG samples were diluted with phosphate-buffered saline (pH 7.2) to a final protein content of 3.0 mg/ml. All samples were stored at −20°C.

Preparation of *E. cuniculi*. *E. cuniculi* were cultured by methods modified from Shadduck (32) and Shadduck et al. (34). Rabbit fibroblasts grown in Earle minimum essential medium were used instead of rabbit choroid plexus cells. Parasites were isolated by rupture of the host cell, filtration, and washing (39).

Peritoneal macrophage preparation. Peritoneal macrophages were collected from female New Zealand White rabbits (average weight, 3 kg) free of encephalitozoonosis as determined by IFAT. Peritoneal exudates were induced by intraperitoneal injection of 200 ml of sterile 2.5% thioglycollate medium without dextrose (Difco Laboratories, Detroit, Mich.). Four to six days later peritoneal exudate macrophages (PEM) were collected by peritoneal lavage. The cells were washed in Hanks balanced salt solution, centrifuged at 225 × *g* for 10 min at 4°C, and resuspended in RPMI 1640 with 25 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; GIBCO Laboratories, Grand Island, N. Y.) supplemented with 10% heat-inactivated fetal calf serum (GIBCO), penicillin (100 U/ml), and streptomycin (100 μg/ml) (complete medium). Cells were washed three additional times in complete medium, and the volumes were adjusted to a final concentration of 10^6 PEM/ml. Cells were dispensed (0.5 ml/chamber) to sterile eight-chamber tissue culture slides (Labtek Products, Naperville, Ill.) and cultured for 2 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Monolayers were rinsed three times with complete medium, and nonadherent cells were aspirated. The adherent PEM were used in phagocytosis assays.

Unstimulated (resident) peritoneal macrophages, collected from uninjected rabbits by peritoneal lavage, were similarly washed, counted, and cultured before use in phagocytosis assays.

Opsonization of parasites. Serum and IgG samples were diluted to give equivalent *E. cuniculi* antibody concentrations (as determined by IFAT). Guinea pig complement (lot no. 3-8475; Microbiological Associates, Bethesda, Md.) was titered and standardized as previously described (6). Suspensions of parasites (10^7/ml) were incubated at 37°C for 1 h with equal volumes of diluted serum (1:100) with or without 10 50% hemolytic complement (CH_{50}) units of guinea pig complement, diluted IgG (1:50) with or without 10 CH_{50} units of guinea pig complement, or complete medium with or without 10 CH_{50} units of guinea pig complement.

Phagocytosis assay. The parasite's small size (2 to 3 μm by 0.5 μm) makes it difficult to visualize and count individual intracellular parasites. Moreover, it is not possible to determine by light microscopy whether intracellular parasites are damaged, killed, or viable. The mere presence of parasites within the macrophage cannot be construed as tantamount to phagocytic killing. In the present study it was important to distinguish between parasites which were internalized yet not killed and parasites which were internalized and subsequently destroyed. Therefore, we designed our phagocytosis assay to measure phagocytic killing of *E. cuniculi* by rabbit peritoneal macrophages.

Opsonized and unopsonized parasites were added to separate peritoneal macrophage monolayers (16 to 21 assays per treatment procedure). Parasite suspensions were similarly added to 16 blank tissue culture chambers not containing peritoneal cells which served as background controls. Cultured macrophages or cell-free controls were incubated for 24 h in a humidified atmosphere at 37°C as before and rinsed three times with a 0.25% (wt/vol) aqueous solution of sodium dodecyl sulfate to lyse peritoneal macrophages and recover parasites. (Treatment with 0.25% sodium dodecyl sulfate did not grossly alter the morphological integrity of parasites.) Parasites were counted with a compound microscope and hemocytometer. Undigested parasites were identified by light microscopy, and the number of undigested parasites per test chamber was derived using the formula: total number of undigested parasites = number of extracellular (i.e., parasites in supernatant) + number of intracellular parasites released by lysis of macrophages.

The number of parasites phagocytosed and killed by PEM was derived by the formula: number of killed

parasites = number of parasites per control chamber background − number of undigested parasites per test chamber.

The percentage of parasites killed by PEM was calculated by the formula: percentage of parasites killed = (number of parasites killed/number of parasites in background control) × 100.

Student's *t* test was employed in the determination of statistical significance of the data.

Scanning electron microscopy. Scanning electron microscopy was employed to determine whether antibody or complement or both enhanced phagocytosis by promoting an increased binding of parasites to peritoneal phagocytes.

PEM were collected and cultured in eight-chamber tissue culture slides as previously described. Suspensions of parasites were incubated (37°C for 1 h) with equal volumes of serum (1:100) with or without 10 CH_{50} units of guinea pig complement or complete medium with or without 10 CH_{50} units of guinea pig complement. Parasite suspensions were then added (0.5 ml) to separate PEM monolayers, incubated (37°C for 1 h), washed with several rinses of complete medium, fixed with 1% buffered (pH 7.2) glutaraldehyde, and processed by standard procedures for scanning electron microscopy.

Transmission electron microscopy. PEM were collected as previously described and plated in Permanox culture plates (Lux Scientific Corp., Thousand Oaks, Calif.) at a concentration of 10^6 PEM/ml. Nonadherent cells were removed with several rinses of complete medium 2 to 3 h later. Secondary lysosomes were labeled with ferritin (Miles Laboratories, Elkhart, Ind.) by exposing PEM monolayers to 0.3 mg of ferritin per ml after the procedures of Jones and Hirsch (17). After 1 to 4 h of incubation with ferritin, the monolayers were washed with several rinses of complete medium.

Suspensions of parasites were incubated (37°C for 1 h) with either immune rabbit serum, normal rabbit serum, or complete medium. PEM monolayers were then infected with a parasite to PEM ratio of 2:1 and incubated for 1 to 6 h. After several rinses with complete medium, the PEM monolayers were fixed with 3% glutaraldehyde, postfixed in osmium, and processed by standard procedures for transmission electron microscopy. Assessment of phagosome-lysosome fusion followed the procedures of Jones and Hirsch (17).

RESULTS

Phagocytosis assays. Normal serum, normal IgG, and complete medium (without rabbit serum) resulted in the destruction of 15 to 18% of the total number of *E. cuniculi* after 24 h of exposure to peritoneal exudate cells (Fig. 1). The presence of immune serum or immune IgG (containing equal concentrations of *E. cuniculi* antibodies as determined by IFAT) in the PEM system resulted in the destruction of 54 and 48% of the parasites. The addition of 10 CH_{50} units of guinea pig complement had little effect on the number of parasites killed when either complete medium or normal IgG was used. The same quantity of complement added to systems containing peritoneal exudate cells plus either immune serum, immune IgG, or normal serum resulted in the killing of 12 to 15% of *E. cuniculi* by 24 h of incubation.

Similar results were obtained when unstimulated peritoneal macrophages were used instead of PEM (Fig. 2). In the presence of normal IgG or complete medium (without rabbit serum), less than 15% of the *E. cuniculi* were phagocytically destroyed. Pretreatment with normal serum enhanced phagocytic killing by 22%. Ten CH_{50} units of guinea pig complement had no effect in the presence of normal serum or complete medium but reduced the number of recoverable parasites in the presence of normal IgG by 6%. As with the PEM, the presence of immune serum or immune IgG resulted in a marked decrease in the number of recoverable *E. cuniculi*. Immune IgG reduced the number by 48%, and immune serum caused a 57% decrease in recoverable parasites. Complement enhanced the effect seen with the immune serum or immune IgG, resulting in a further reduction of recoverable parasites by 12 and 23%, respectively.

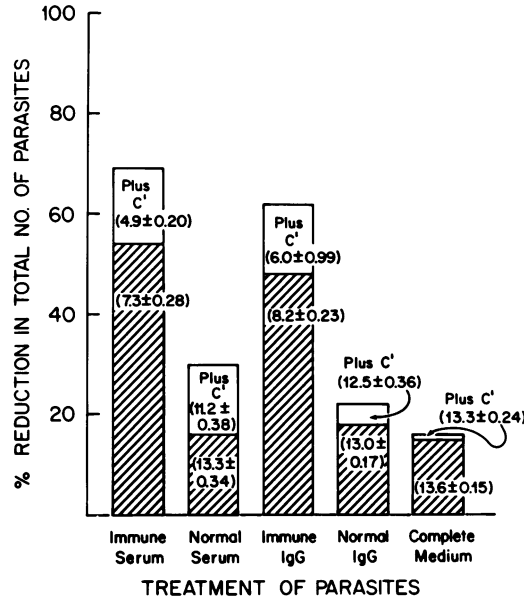

FIG. 1. *PEM: Effect of serum, IgG, and complement on the removal of E. cuniculi from cultures of rabbit PEM. Mean number (×10^5) of parasites recovered per assay chamber (N = 16 to 21) is in parentheses. Percent reduction was derived by comparing the mean number of parasites recovered from each treatment group with the mean number of parasites recovered in the background control (= 16 × 10^5).*

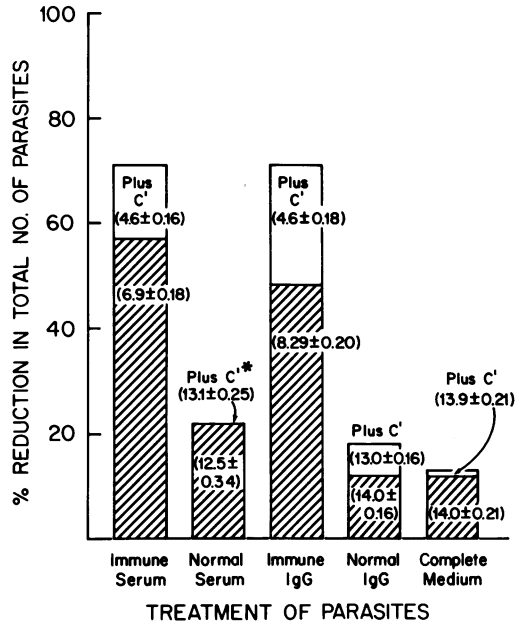

FIG. 2. *Unstimulated peritoneal macrophages: effect of serum, IgG and complement on the removal of E. cuniculi from cultures of rabbit peritoneal macrophages. Mean number ($\times 10^5$) of parasites recovered per assay chamber (N = 16 to 21) is in parentheses. Percent reduction was derived by comparing the mean number of parasites recovered from each treatment group with the mean number of parasites recovered in the background control (= 16×10^5). *, 18* reduction.*

PEM were slightly more effective than unstimulated peritoneal macrophages in killing parasites treated with normal IgG or parasites treated with guinea pig complement and normal serum, normal IgG, or complete medium. In all other cases PEM and unstimulated peritoneal macrophages were equally efficient in phagocytosing *E. cuniculi*.

Binding of opsonized parasites (scanning electron microscopy). Consistent trends were observed in the binding of parasites to peritoneal macrophages (statistical data not listed). Small numbers of parasites treated with normal serum (Fig. 3A) or complete medium were bound to peritoneal macrophages. Treatment of parasites with guinea pig complement and normal rabbit serum did not promote additional binding (Fig. 3B). By contrast, numerous immune serum-treated parasites bound and formed rosettes with peritoneal macrophages (Fig. 3C). Treatment of parasites with immune serum supplemented with guinea pig complement did not markedly enhance the extensive binding seen with immune serum-treated parasites (Fig. 3D). Approximately the same number of parasites were seen binding to peritoneal macrophages in both treatment groups (i.e., antibody plus complement or antibody alone). Parallel transmission electron microscopy studies revealed a close correlation between the number of parasites bound and the number of parasites internalized.

Lysosomal fusion (transmission electron microscopy). Within 2 to 6 h, ferritin-labeled lysosomes were seen fusing with phagocytic vacuoles containing parasites treated with immune rabbit serum (Fig. 4). Evidence of phagosome-lysosome fusion was present in the vast majority of peritoneal macrophages containing immune serum-treated parasites. Within the peritoneal macrophages the ferritin label formed a contiguous boundary circumscribing the parasites treated with immune rabbit serum. Parasites treated with normal rabbit serum or complete medium were internalized in smaller numbers. There was no evidence of fusion of ferritin-labeled lysosomes with phagosomes containing parasites treated with normal rabbit serum or complete medium (Fig. 5).

DISCUSSION

Phagocytosis has long been regarded as a mechanism of resistance to many unicellular pathogens. However, endocytosis of protozoan parasites is not tantamount to phagocytic killing. For example, mouse macrophages can endocytose *Toxoplasma gondii* trophozoites; however, phagosomes are unable to fuse with lysosomes (17) unless extracellular trophozoites are opsonized with antitoxoplasma serum (2, 35). Opsonization by IgG and complement components bound to the surface of bacteria stimulate bactericidal activity of phagocytes by stimulating the production of the bactericidal agents H_2O_2 (9, 13–16, 29, 37) and the release of lysozyme and β-glucuronidase (14, 15). Even if increased synthesis and release of lysosomal enzymes and peroxides occurs, it may be ineffective in the intracellular destruction of endocytosed parasites. For example, *Leishmania* are impervious to lysosomal enzymes and are able to proliferate within the macrophage lysosome (7). Other parasites escape from the phagosomes before phagosome-lysosome fusion and thereby evade exposure to lysosomal enzymes (9, 21, 30).

The results of the present study suggest that *E. cuniculi* behaves in a manner similar to *T. gondii*. Parasites were capable of infecting and residing within peritoneal phagocytes. Within the phagocyte the parasites escaped from the lysosomes. Treatment with normal serum, normal IgG, guinea pig complement, or complete medium had little effect on the uptake of para-

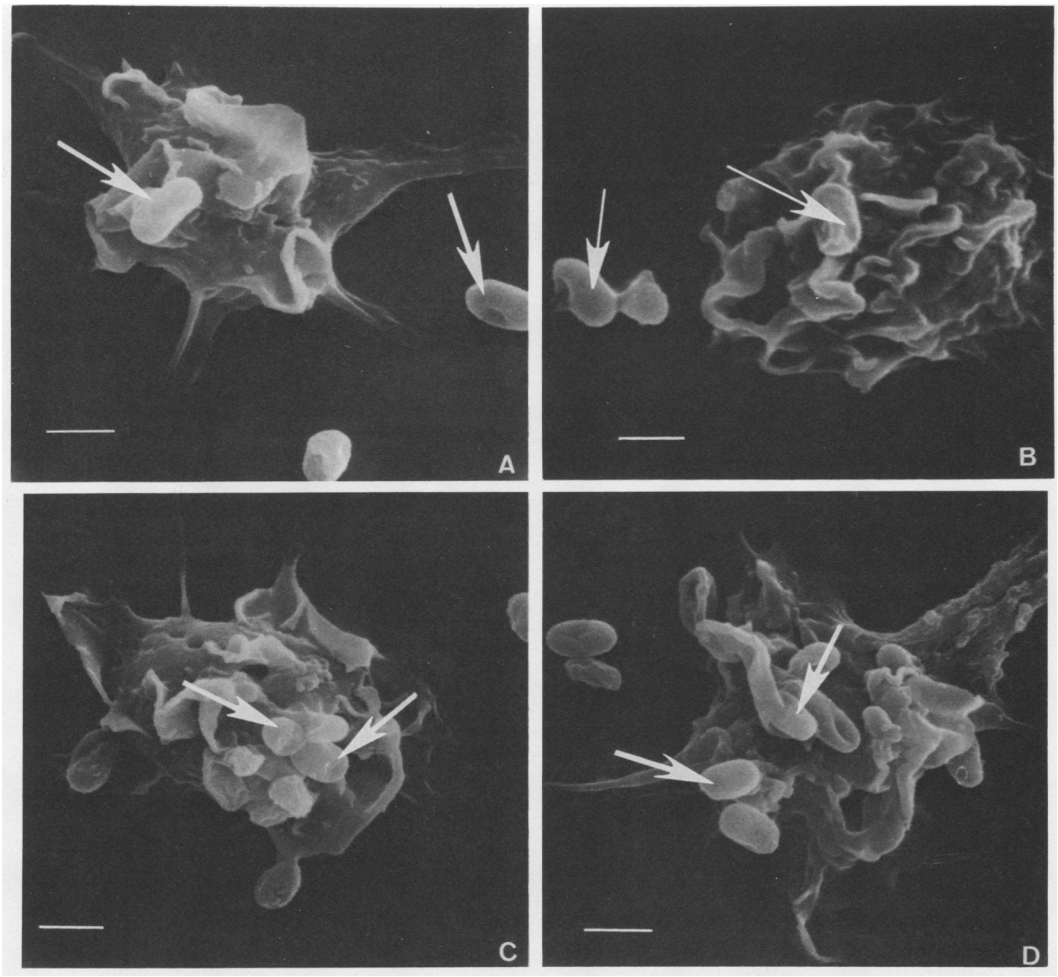

FIG. 3. *Binding and early stages of endocytosis of E. cuniculi by rabbit peritoneal macrophages. Parasites (arrows) were pretreated as described in Materials and Methods. (A) Sparse binding of parasites pretreated with normal rabbit serum. (B) Pretreatment of parasites with normal rabbit serum supplemented with guinea pig complement did not enhance binding. (C) Large numbers of parasites pretreated with immune rabbit serum were bound to and formed "rosettes" with peritoneal macrophages. (D) Extensive binding of parasites pretreated with immune rabbit serum supplemented with guinea pig complement. Each bar represents one micrometer.*

sites by rabbit mononuclear peritoneal cells while treatment with immune serum or immune IgG greatly increased phagocytosis. It has been demonstrated that *E. cuniculi* readily grows in parasitophorous vacuoles of infected mouse macrophages and that lysosomes do not fuse with the vacuole (38). However, our results suggest that specific antibody either damages or alters extracellular parasites and thereby promotes the fusion of parasite-containing phagosomes with lysosomes in a manner similar to the condition with *T. gondii* (2, 35). Alternatively, it is possible that specific antibody has an innocuous effect on extracellular *E. cuniculi*, but once these antibody-coated parasites are internalized they are susceptible to intracellular hydrolysis.

The role of human monocyte Fc and C3 receptors in the binding and endocytosis of antibody and complement-treated particles has been established by Ehlenberger and Nussenzweig (10) who found that IgG promoted binding and endocytosis of sheep erythrocytes. Addition of C3 greatly increased binding, but in the absence of IgG did not stimulate internalization of sheep erythrocytes.

Our results are compatible with these obser-

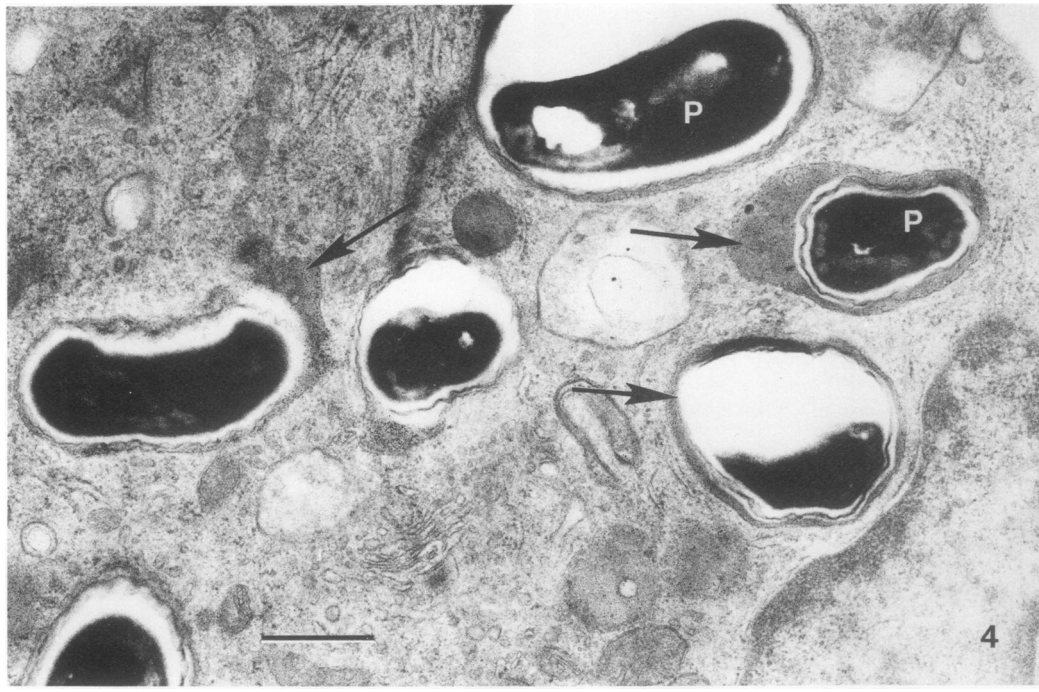

FIG. 4. *Phagosome-lysosome fusion promoted by immune serum. Phagosomes containing parasites (P) pretreated with immune rabbit serum were seen fusing with ferritin-labeled lysosomes. Arrows show dense ferritin label circumscribing the parasites. Bar represents 1 μm.*

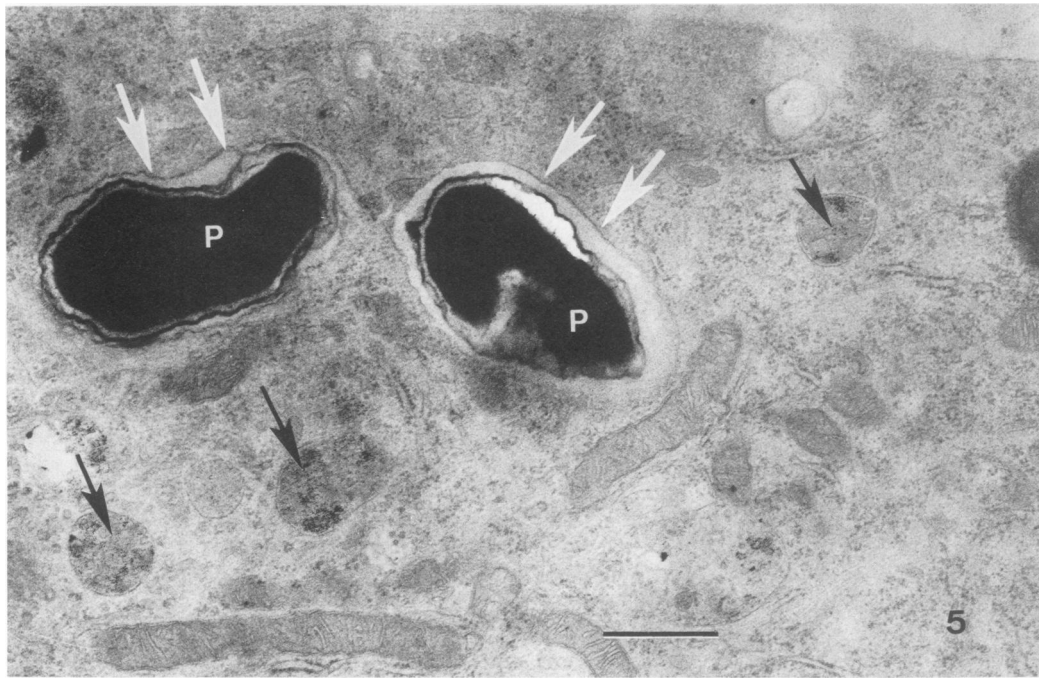

FIG. 5. *Absence of phagosome-lysosome fusion. Parasites (P) pretreated with normal rabbit serum were enclosed in a vacuole (white arrows) which did not fuse with ferritin-labeled lysosomes (black arrows). Bar represents 1 μm.*

vations. Opsonization by immune serum was predominantly due to IgG antibodies. Addition of complement significantly augmented phagocytosis of *E. cuniculi* in the presence of antibody. The scanning electron microscopy studies suggest that this augmentation was due, at least in part, to enhanced binding of opsonized parasites via C3 and Fc receptors on the peritoneal phagocyte surface. Subsequent examination by transmission electron microscopy revealed that the number of parasites internalized correlated with the number of parasites bound to the macrophage cell membrane.

The physiological state of macrophages can influence phagocytosis. Macrophages collected from mice injected intraperitoneally with thioglycollate medium are larger, contain higher concentrations of lysosomal enzymes, and spread more rapidly than normal tissue macrophages (1, 36). Moreover, thioglycollate-induced macrophages are capable of ingesting complement-coated erythrocytes, whereas normal peritoneal macrophages bind but do not ingest complement-coated erythrocytes (4). We found that, with few exceptions, unstimulated and thioglycollate-induced mononuclear peritoneal cells were equally effective in the phagocytosis of *E. cuniculi*.

The fact that encephalitozoonosis is asymptomatic and rarely fatal (33) suggests that mechanisms exist to control the multiplication of the parasite in vivo. Protection is not complete since chronic infections are common (33) and latent encephalitozoonosis can be activated by hydrocortisone (5). Treatment with antibody and complement does not significantly reduce infectivity of *E. cuniculi* to cultured rabbit fibroblasts (unpublished data). In the absence of antibody, parasites grow within parasitophorous vacuoles of macrophages (38). We suggest that, in the presence of antibody, macrophages are capable of destroying *E. cuniculi*. This phagocytic killing is potentiated by specific (antibody) and nonspecific (complement) humoral factors. Ancillary immune modalities, such as cytotoxic T-lymphocytes and lymphokines, might function independently or in concert with macrophages in the control of encephalitozoonosis.

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