INFECTION AND IMMUNITY, Feb. 1971, p. 278-286

Vol. 3, No. 2
Printed in U.S.A.

Immunological Study on the Host Cell Penetration Factor of *Toxoplasma gondii*

RAGNAR NORRBY

Department of Virology, Institute of Medical Microbiology, University of Gothenburg, Sweden

Received for publication 21 September 1970

The host cell penetration factor (PEF) of *Toxoplasma gondii* was studied by biochemical and immunological techniques. Sephadex gel filtration of an ammonium sulfate-precipitated PEF yielded two components with different molecular weight, but both having penetration-enhancing activity. The methods for purification removed at least 99.9% of extraneous protein. For demonstration of a significant enhancement of penetration, 0.001 μg of protein was sufficient. Biochemically, they appeared to be acid proteins with the same electrophoretic mobility. Both components showed maximal enhancement of penetration at *p*H 7.6 and 37 C. PEF antisera reduced the penetrative capacity of *Toxoplasma* parasites. The penetration-enhancing effect of the two components of PEF was inhibited by antiserum against any of them. Moreover, immunologically identical immunoprecipitates were obtained when antiserum reacted with the two components. The results thus indicated that the two components of PEF were immunogenically identical and that the difference in molecular weights might result from aggregation. Immunofluorescence indicated that PEF was related to cytoplasmic structures located in the anterior end of *Toxoplasma*. A possible relation between these structures and the paired organelle or the convoluted tubes was discussed. The number of parasites with immunofluorescence was low shortly after host cell penetration and increased during the intracellular life of the parasites after kinetics, previously observed for synthesis of PEF as well as for lysosomal activity of *Toxoplasma*.

Toxoplasma gondii contains a factor important for the penetrative capacity of the *Toxoplasma* trophozoites (11, 15). The factor, extractable from suspensions of parasites, seems to be of an enzymatic nature, probably of lysosomal origin (14, 15). It is spent by the parasites during the process of penetration, exerting its effect upon the host-cell wall, and again synthesized during the intracellular life of the parasite (13). If added to parasite inocula, it increases the virulence for mice of various *Toxoplasma* strains (12). In the present report, the penetration-enhancing factor (PEF) of *Toxoplasma* is studied by means of immunological and biochemical techniques.

MATERIALS AND METHODS

Toxoplasma parasites. The RH strain of *T. gondii* was used in all experiments. Parasites were collected from the peritoneal cavity of Swiss albino mice on the 3rd day after intraperitoneal infection. Suspensions of parasites, free from exudate fluid and from most of the peritoneal cells, were prepared according to a technique described previously (10). These suspensions were used as test parasites in the assays of parasite penetration and as inoculum for cell cultures.

Test for assaying penetration of Toxoplasma parasites. The cell culture technique used was described previously (10). Materials to be tested or Hanks balanced salt solution (BSS) were mixed with test parasites and inoculated into test cultures. After incubation at 37 C for 19 hr, the cultures were read in a phase-contrast microscope. The quotient between the number of *Toxoplasma* parasites that had penetrated host cells and the number of exposed cells was determined and this quotient × 100 is referred to as the relative number of infective units (RNIU).

Parasite-infected suspension cell cultures. HeLa suspension cell cultures containing 2.4×10^8 cells in 200 ml of Eagles minimal essential medium for suspension cultures (Grand Island Biological Co., Grand Island, N. Y.) with penicillin and streptomycin (100 units and 100 μg, respectively), double amounts of amino acids, and 10% human dye-test negative serum were used. A cell culture was inoculated with 25 ml of a suspension of *Toxoplasma* parasites containing 2.5×10^6 parasites per ml and incubated at 37 C for 48 hr. Cells and extracellular parasites were then sedimented by centrifugation at 400 × *g* for 20 min, suspended in 35 ml of BSS, and inoculated into two suspension cultures which were incubated at 37 C for 2 days. The 70-ml amount of cell-parasite suspension obtained was used as inoculum for another four

cultures. This second passage resulted in a 140-ml suspension of parasite-infected cells and extracellular parasites and was used for extraction of the PEF.

Extraction of PEF. The technique for extraction of PEF has been described in detail previously (15). Briefly, the material was frozen and thawed three times, sonically treated for 15 min, and centrifuged at 20,000 × *g* for 45 min. The supernatant obtained was incubated at 37 C for 1 hr and centrifuged at 80,000 × *g* for 45 min to remove substances inhibiting PEF. After dialysis against distilled water for 4 hr, the material was lyophilized and stored frozen at −90 C. The lyophilized PEF was dissolved in BSS (2.2 mg of protein per ml). This was considered a standard solution of PEF and submitted to fractionation and other biochemical treatments of PEF. All preparatory work with PEF was performed at temperatures below 5 C.

Gel filtration. Sephadex G-200 (Pharmacia, Uppsala, Sweden) was used. The column size was 90 by 2.5 cm and the flow rate was 5 ml per 50 min. A 5-ml amount of BSS, containing ammonium sulfate-precipitated PEF, was added to the column and eluted with BSS. Fractions of 5 ml were collected, and the extinction at 280 nm was measured in a Beckman DB spectrophotometer. Each fraction or pool of 2 to 6 fractions was tested for PEF.

Isotope labeling of HeLa cells. To a 200-ml HeLa suspension cell culture, 500 μCi of tritium-labeled glycine (glycine-*2-T*, data sheet 7631, The Radiochemical Center, Amersham, England) was added, and the culture was incubated at 37 C for 48 hr. The cells were sedimented by centrifugation at 440 × *g* for 20 min, resuspended in 17.5 ml of BSS, and subsequently mixed with 17.5 ml of a parasite-cell suspension from a parasite-infected culture incubated and treated as described above. PEF was extracted from the preparation, precipitated with ammonium sulfate (30% saturation), and gel filtered. During the preparatory work, samples were drawn and the radioactivity was assayed in a Packard liquid scintillator after mixing 0.5 ml of the sample to be tested with 9.5 ml of Brays solution (2).

Treatment with trypsin and phenol. Three milliliters of a 2.5% solution of crystallized trypsin (Boehringer & Soehne GmbH, Mannheim, Germany) in phosphate-buffered saline (*p*H 7.6) was mixed with 1.5 ml of PEF and incubated at 37 C for 3 hr. The treatment with phenol was performed by mixing 1.5 ml of an 80% (w/w) solution of phenol with 1.5 ml of PEF and incubating the mixture at 37 C for 3 hr. The bottom phase obtained after centrifugation of the phenol-treated PEF at 1,100 × *g* for 30 min and the trypsin-PEF mixture were both precipitated twice with 30% saturated ammonium sulfate. The precipitates, then obtained, were dissolved and tested for residual PEF. BSS mixed with trypsin or phenol was treated as described above and served as controls, as did PEF twice-precipitated with ammonium sulfate.

Protein determination. Quantitative protein determination was performed by the technique described by Lowry et al. (9).

Antisera. Antisera were prepared by immunization against various fractions of gel-filtered PEF (Table 4). The volume of each fraction was adjusted to 10 ml by dialysis against polyethyleneglycol. In addition, an anti-HeLa cell serum was prepared by immunization with a suspension of homogenized HeLa suspension cells. One rabbit and 12 white rats were injected with the fractions of PEF. All animals were bled before the immunization and the preimmune sera obtained were tested for absence of dye-test and *Toxoplasma* complement fixation (CF) antibodies. The animals received various numbers of intramuscular and intraperitoneal injections (Table 4), each consisting of portions of the antigen and Freunds complete adjuvant (Difco) and 100 μg of polyI-polyC in 0.1 ml of saline (3; Microbiological Associates, Bethesda, Md.). The injections were given with 2-week intervals, and the animals were bled by heart puncture 1 week after the last injection. The sera were tested with dye tests, CF tests, immunofluorescence, gel diffusion, and *Toxoplasma*-penetration assays. If not otherwise stated, the sera were used diluted 1:2 in BSS and inactivated at 56 C for 30 min.

Dye test. Dye tests were performed by the method of Sabin and Feldman (16) with the modification described by Strannegård (17). The final dilutions of the sera were 1:2.5, 1:10, and 1:50.

CF tests. The sera were tested for CF antibodies against 4 CF units of an antigen obtained from *Toxoplasma*-infected hen's eggs. A guinea pig serum was used as source of complement and was diluted 1:55. The sera were diluted 1:4, 1:8, 1:16, and 1:32 (final dilutions).

Immunodifiusion tests. Double diffusion in gel was performed by the micromodification of Wadsworth (18). In the immune electrophoretic studies, the technique described by Wadsworth and Hanson (20) was used. Electrophoreses were run for 45 min with a field strength of 5 v per cm. Photographic registration was made with Polaroid panchromatic film reproduction (19).

FA test. The indirect fluorescent-antibody (FA) technique was used. HeLa cells cultivated for 24 hr in Leighton tubes were each inoculated with 10^6 parasites in 1 ml of BSS. After various times of incubation at 37 C, the cover slips were removed, washed in BSS, air-dried, and fixed in acetone for 10 min at room temperature. Samples of the parasite inoculum were prepared by air drying 0.15-ml amounts and fixing in acetone as above. In all experiments, a rabbit anti-PEF serum was tested. The samples were incubated for 30 min with the serum, washed five times in saline, and incubated for 30 min with a fluorescein isothiocyanate-conjugated, dye-test negative, anti-rabbit globulin of sheep origin (Progressive Laboratories, Baltimore, Md.), diluted 1:2 in saline. After five washings in saline, the samples were counterstained in Evan's Blue, 1:10,000 in distilled water, washed in running tap water, dried, and mounted in Elvanol. A dye-test positive serum from rabbit and a preimmune serum were used as controls. To exclude any subjective influence of the reader, series of experiments were performed on coded samples.

RESULTS

Purification of PEF. Standard solutions of PEF were subjected to ammonium sulfate precipitation and gel filtration. To 1.8 g of lyophilized PEF, containing 220 mg of protein and dissolved in 100 ml of BSS, 15 g of crystallinic ammonium sulfate was added to give a saturation of 25%. The precipitate formed was sedimented by centrifugation at 1,100 × *g* for 10 min, and the supernatant was carefully pipetted off. The sediment was dissolved in 5 ml of BSS. Two more precipitates formed at a saturation of 30 and 35% of $(NH_4)_2SO_4$, respectively, were collected from the standard solution and dissolved as described above. The dissolved precipitates and the resulting supernatant were all dialyzed against BSS for 4 hr and then tested for content of PEF and protein. All activity of PEF was found in the precipitate formed at 30% saturation. This material contained less than 3% of the initial protein concentration (Table 1).

The precipitate formed with 30% saturated ammonium sulfate was gel filtered on Sephadex G-200. The fractions collected were tested for adsorption at 280 nm and for penetration-enhancing activity. The PEF was found in two distinctly separated peaks (designated 1 and 2) not coinciding with any of the major peaks of the spectrophotometry (Fig. 1).

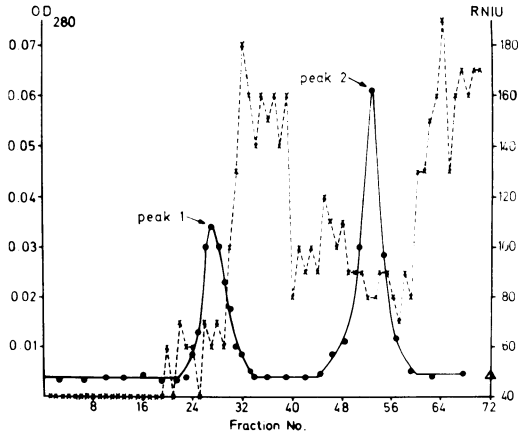
This pattern was encountered in all of a great number of batches of PEF processed. It was therefore interpreted as indicating the presence of two components with different molecular weights, both with an enhancing effect on the penetration of *Toxoplasma* parasites, and it seemed as if these components constituted only a very small part of the protein content of the PEF standard solution. Moreover, purification of PEF resulted in a near 100% increase in penetration-enhancing activity (Table 1). Peak 1 and peak 2 contained less than 0.2% of the proteins present in the standard solution of PEF, whereas the penetration-promoting activity per microgram of protein was increased about 2,500 times.

Table 2 demonstrates, in another way, the capacity of the purification methods to remove the extraneous protein. HeLa cells incubated with tritium-labeled glycine were mixed with HeLa

TABLE 1. *Purification of penetration-enhancing factor (PEF) by ammonium sulfate precipitation and gel filtration*[a]

Sample	Protein concn (μg per ml)	PPA per ml of undiluted sample	PPA per μg of protein
PEF (standard solution)	2,200	3,480	1,58
Precipitate	59	3,384	57.36
Peak 1	1.5	3,312	2,208.00
Peak 2	1.8	3,368	1,871.11

[a] Protein contents of a standard solution of PEF, the precipitate formed at 30% saturation with $(NH_4)_2SO_4$, and samples corresponding to the two peaks of activity obtained by gel filtration were assayed. Aliquots of the samples were then diluted 1:80 and tested for penetration-enhancing effect. The penetration-promoting activity (PPA) per milliliter of undiluted sample and per microgram of protein was determined.



[1] FIG. 1. *Gel filtration of PEF. RNIU values* (●) *and optical density at 280 nm* (×) *of 5-ml fractions eluted from a Sephadex G-200 column. RNIU value of the BSS control* (△) *is plotted at the right ordinate.*

TABLE 2. *Efficacy of purification of penetration-enhancing factor (PEF) by ammonium sulfate precipitation and gel filtration*[a]

Material	Amt (ml)	Counts per minute	Per cent
PEF extract	20	6,439 ± 147	100
$(NH_4)_2SO_4$ precipitate	5	1,455 ± 17	6.02
Peak 1	5	22 ± 0.8	0.07
Peak 2	5	8 ± 0.1	0.02
BSS[b]		3	
Bray's solution		3	

[a] Radioactivity in counts per minute of PEF extracted from a mixture of *Toxoplasma*-infected HeLa cells and cells labeled with tritiated glycine, the $(NH_4)_2SO_4$ precipitate of such an extract, two fractions (peak 1 and 2) obtained by gel filtration, and the diluents used is given. Results are expressed as mean values ± σ based on four assays and in per cent of the activity of the untreated extract.

[b] Hanks balanced salt solution.

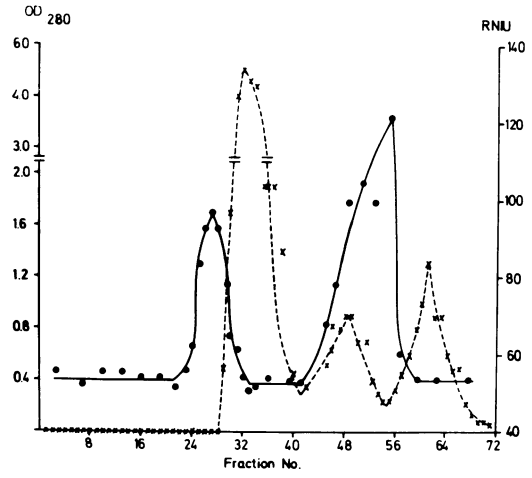


FIG. 2. *Estimation of molecular weight of PEF. RNIU values (●) and optical density at 280 nm (×) of 5-ml fractions obtained by gel filtration of human serum mixed with PEF.*

TABLE 3. *Effect of phenol and trypsin on penetration-enhancing factor (PEF)*[a]

Additive to test parasites	Treatment	RNIU[b]
BSS		49.3
BSS	Trypsin + $(NH_4)_2SO_4$	49.7
PEF	$(NH_4)_2SO_4$	64.7
PEF	Trypsin + $(NH_4)_2SO_4$	49.7
BSS	Phenol + $(NH_4)_2SO_4$	48.4
PEF	Phenol + $(NH_4)_2SO_4$	48.7

[a] After treatment of PEF with trypsin or phenol, it was precipitated with $(NH_4)_2SO_4$ and assayed for penetration-enhancing effect. Balanced salt solution (BSS) was used in controls.

[b] Relative number of infective units.

cells infected with *Toxoplasma*. PEF was extracted, and samples were drawn from the extract before ammonium sulfate precipitation, from the dissolved precipitate obtained with 30% $(NH_4)_2SO_4$, and from peaks 1 and 2 eluted from the Sephadex column. The results indicated that the combination of ammonium sulfate precipitation and Sephadex chromatography reduced the amount of HeLa cell proteins containing glycine to less than 0.1% of that found before fractionation of PEF.

Some physico-chemical characteristics of PEF. Results of gel filtration of a mixture of 2.5-ml samples of ammonium sulfate-precipitated PEF and human serum suggested a molecular weight of the peak 1 component ranging between those of immunoglobulins M and G (700,000 to 1,000,000), whereas the peak 2 component appeared to be of an order of magnitude between that of immunoglobulin and the albumin, i.e., within 70,000 to 150,000 (Fig. 2).

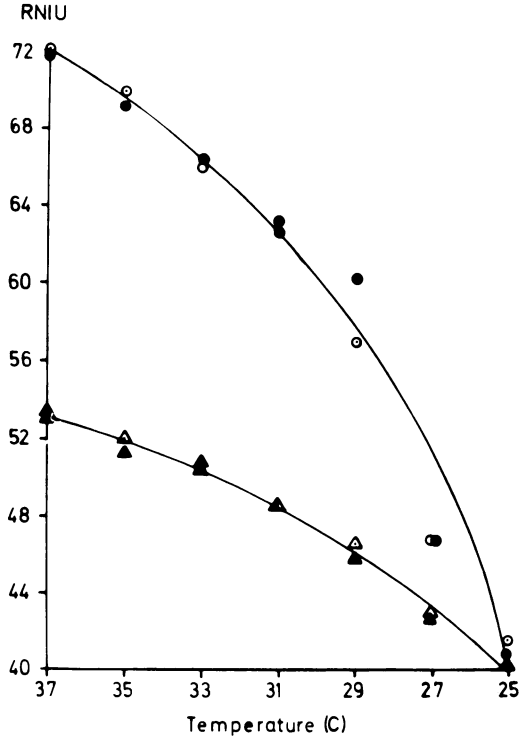


FIG. 3. *Temperature optimum of PEF. A standard solution of PEF was fractionated by Sephadex chromatography; the peak 1 (○) and peak 2(●) fractions were tested for penetration enhancement at various temperatures. RNIU values obtained for these groups and for the BSS controls (△ and ▲, respectively) are plotted against incubation temperatures.*

The findings that treatment with trypsin or phenol destroyed the activity of PEF strongly supported the view that PEF was of protein in nature. In the experiments, PEF was incubated with 1.25% trypsin or 80% phenol for 3 hr. To remove the trypsin and phenol after the treatments, PEF was precipitated with ammonium sulfate before the assays in cell cultures (Table 3).

Peak 1 and 2 materials were tested for the stability of the penetration-promoting effect at various temperatures and *p*H levels as well as optimal temperature for penetration enhancement. No difference was found between the two components, both showing maximal stability at *p*H 7.6 and increasing instability at temperatures above 37 C, i.e., in these respects they did not differ from the PEF of nonpurified extracts (11).

TABLE 4. *Antigens and animals used for preparation of immune sera*

Antigen		Protein (μg/ml)	Animal	Injections (im)[b]		Injections (ip)[b]		Designation of antiserum
Designation	Material[a]			No.	Vol. (ml)	No.	Vol. (ml)	
I	Fractions eluted before peak 1	10–25	Rat	3	1	4	1	A
II	Peak 1	15–35	Rat	3	1	4	1	B
III	Fractions eluted between peak 1 and peak 2	15–40	Rat	3	1	4	1	C
IV	Peak 2	10–30	Rat	3	1	4	1	D
V	Fractions eluted after peak 2	20–55	Rat	3	1	4	1	E
VI	Pool of peak 1 and 2	20–55	Rabbit	2	2	3	2	F
VII	Homogenized HeLa cells	50	Rat	3	1	4	1	G

[a] Each antigen was mixed with Freund's complete adjuvant and 100 μg of polyI-polyC.
[b] Antigens were administered by intramuscular (im) or intraperitoneal (ip) routes.

TABLE 5. *Effect of antisera on the enhancement of the Toxoplasma penetration*[a]

Antigen used	RNIU values of antiserum[b]							
	A	B	C	D	E	F	G	BSS
II	68.3	53.1	67.9	51.8	68.1	52.8	68.3	68.2
IV	67.3	52.2	68.8	53.0	68.0	52.1	68.6	68.2
VI	69.4	53.0	68.9	53.4	67.5	50.8	68.5	67.2
BSS	51.5	52.1	51.8	51.9	51.7	52.1	51.9	51.8

[a] Antigen and antiserum were mixed and incubated for 1 hr at room temperature; the mixtures added to test parasites were inoculated into cell cultures. Antigens II and IV were used in dilution 1:1,250, antigen VI in 1:2,500, and the sera in 1:2. Dilutions were made with balanced salt solution (BSS).
[b] Penetration of *Toxoplasma* is assayed by the relative number of infective units (RNIU), and enhancement is revealed by increased values relative to those of the control, in which antigen or antiserum (or both) is replaced by BSS.

At temperatures below 37 C, both peaks gradually lost their activity; at 25 C, no penetration enhancement was seen (Fig. 3).

Effect by PEF antisera on the host cell penetration of Toxoplasma. The antigens used and the immunization schedule for preparation of antisera are described in Table 4. Preimmune sera as well as antisera were tested by dye tests and CF tests. None of these tests showed presence of antibodies against *Toxoplasma*.

Subsequently, the antisera were tested for content of antibodies against PEF. As antigens, the highly potent fractions II (peak 1) and IV (peak 2) were used, separately or in combination (VI). The antigens diluted is BSS and the antisera were allowed to react for 1 hr at room temperature, and the mixtures were then added to test parasites and inoculated into test cultures.

Table 5 demonstrates that the penetration-enhancing effect of the antigens could be removed by the homologous antisera (B, D, and F) and, in addition, that the inhibiting antisera cross-reacted with all of the three enhancing antigens. As antigen VI was a combination of II and IV, the results suggested an antigenic similarity between these two antigens. Neither the antisera against antigens I, III, V, or VII nor the preimmune sera showed inhibiting effect on the enhancement of the *Toxoplasma* penetration. In a following experiment, antisera B and D were serially diluted and the dilutions were tested for inhibiting effect on antigens II and IV. In agreement with the results mentioned above, it was found that the sera inhibited both antigens to the same extent (Fig. 4).

The antisera B and D were also found to be inhibitory to the penetration of ordinary parasites, not stimulated by addition of PEF. In three experiments, test parasites were incubated with serum for 1 hr before the inoculation into test cultures. Table 6 shows that the penetrative capacity of the parasites incubated with the antisera B, D, or F was reduced, whereas this could not be demonstrated when the other antisera or the preimmune sera were tested. Moreover, addition of the inhibiting antisera to test cultures 19 hr after the inoculation of the test parasites resulted in a markedly reduced penetrative capac-

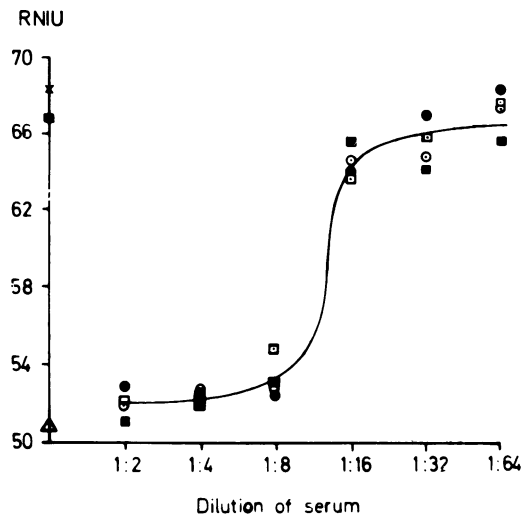


FIG. 4. *Cross-reactions between anti-PEF sera. Antigen II (open symbols) or IV (dark symbols) was mixed with various dilutions of antiserum B (circles) or D (quadrates), incubated at room temperature for 1 hr, mixed with test parasites, and inoculated into test cultures. RNIU values obtained are plotted against serum dilutions. Controls (BSS, △, antigen II, □, and antigen IV, ×; without serum) are plotted at the ordinate.*

ity of parasites then released in the cultures by bursting host cells (Table 7). The observations were interpreted as indicating that activity of PEF was important for the normal penetration of the *Toxoplasma* parasites.

Diffusion-in-gel studies. The preparations of PEF obtained by Sephadex chromatography, an HeLa cell homogenate, antiserum F prepared by immunization of a rabbit with antigen preparation VI, and a rabbit *Toxoplasma* hyperimmune serum with a dye-test titer of 1:16,000 were studied with the double diffusion-in-gel technique. The antigens were used in a concentration corresponding to 50 times the one of the PEF standard solution, and the antisera were used two times concentrated. Immunoprecipitates were demonstrable testing antiserum F against the antigens II, IV, and VI. No precipitates appeared with any of the other antigens tested or the *Toxoplasma* hyperimmune serum (Fig. 5). The precipitates obtained showed a complete coalescence, forming one continuous precipitation line. Thus, preparations II, IV, and VI seemed to contain one and the same precipitinogen which was absent in the other preparations and which did not originate from HeLa cells. Antibodies against this precipitinogen were not present in a *Toxoplasma* hyperimmune serum in a concentration detectable by means of the technique used. These

TABLE 6. *Effect of antisera on the penetration of Toxoplasma parasites*[a]

Expt	RNIU values of serum[b]		
	Designation	Preimmune	Immune
1	A	ND	52.8
	C	ND	52.0
	E	ND	53.6
	G	ND	52.1
	BSS	49.3	49.3
2	B	51.8	37.3
	D	51.9	37.6
	BSS	51.6	51.6
3	F	53.5	36.7
	BSS	52.5	52.5

[a] Test parasites and serum, diluted 1:2 in Hanks balanced salt solution (BSS), were mixed, incubated for 1 hr at room temperature, and inoculated into test cultures. Parasites mixed with BSS or preimmune serum were used as controls.

[b] Penetration of *Toxoplasma* is assayed by relative number of infective units (RNIU). ND, not done.

TABLE 7. *Effect of antisera on the penetration of Toxoplasma parasites produced in the infected cell culture*[a]

Additive	RNIU values at[b]	
	19 hr	25 hr
Hanks balanced salt solution	51.2	92.2
Preimmune serum (B)	51.2	93.1
Preimmune serum (D)	51.0	92.8
Preimmune serum (F)	51.7	90.5
Antiserum B	51.1	67.1
Antiserum D	51.2	69.7
Antiserum F	51.5	65.1

[a] Nineteen hours after inoculation of parasites, an antiserum, preimmune serum, or Hanks balanced salt solution (1:2 dilution) was added to the cultures.

[b] Penetration was assayed by relative number of infective units (RNIU) determined at 19 and 25 hr after inoculation of the cultures.

results were consistently obtained with several batches of antigen studied. In immunoelectrophoresis, one antigenic component with a mobility between the β_2 and γ globulins was demonstrable. The precipitinogen migrated at the same rate as PEF, suggesting a molecular identity between the two (Fig. 6).

Immunofluorescence studies. Attempts to localize the particular structures of the *Toxoplasma*

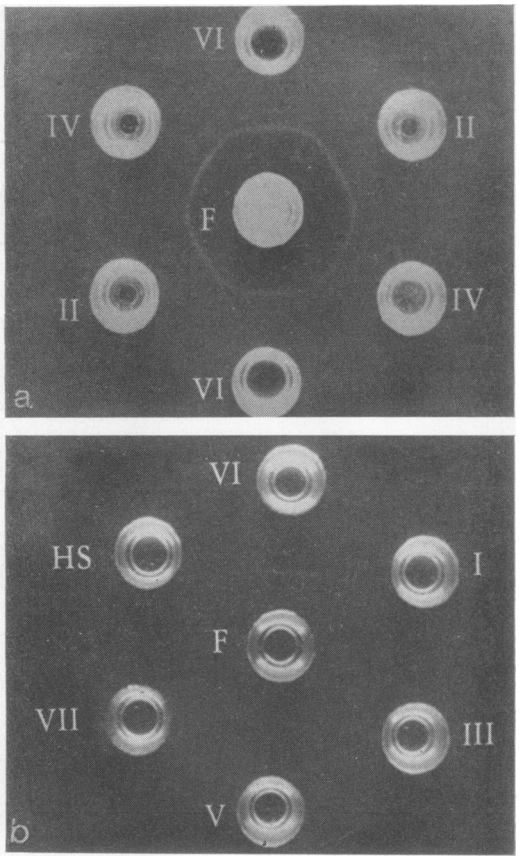


FIG. 5. *Diffusion-in-gel studies of PEF. Immunoprecipitates obtained when antigen I-VII (see Table 4) were tested against antiserum F or a Toxoplasma hyperimmune serum (HS). All antisera were used 2× concentrated. Photographic registration was performed on the 3rd day.*

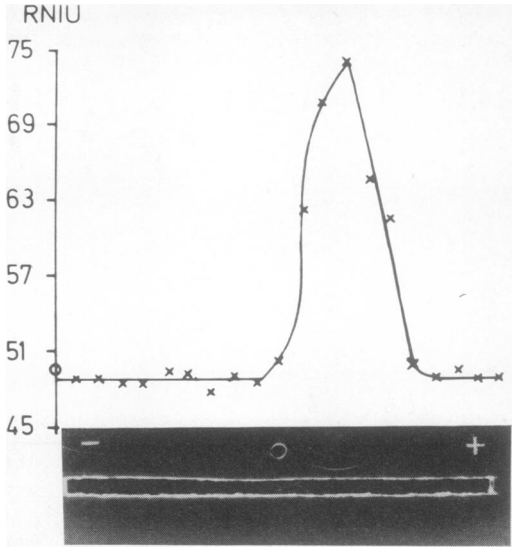


FIG. 6. *Gel electrophoresis of PEF. PEF was added to two circular basins in an agar gel, and electrophoresis was run for 45 min with a field strength of 8 v per cm. Anti-PEF serum was added to a rectangular basin cut out below one of the antigen basins. On each side of the other antigen basin, 10 3-mm pieces were cut out, eluted in BSS, and tested for penetration-enhancing activity. The RNIU values assayed are plotted against the electrophoretic motility of the immunoprecipitinogen in the PEF preparation. The RNIU value of the BSS control is plotted at the ordinate.*

parasites associated with PEF were done by means of the indirect FA technique, by using the rabbit antiserum F. Cover slips with parasite-infected HeLa cells, incubated for various times, were fixed, incubated with antiserum and, subsequently, with a fluorescein isothiocyanate-conjugated antirabbit globulin. Fixed parasites of the inoculum used for infecting the cell cultures were treated in the same way. Samples incubated with preimmune serum, *Toxoplasma* hyperimmune serum, or BSS instead of antiserum F were used as controls.

Parasites incubated with anti-PEF serum showed a specific immunofluorescence, entirely different from that observed with the dye-test positive serum (Fig. 7). With the use of *Toxoplasma* hyperimmune serum, the fluorescence was restricted to the pellicle of the parasite. With antiserum F, on the other hand, both parasites of the inoculum and those inside infected cells revealed small cytoplasmic fluorescent spots in the anterior end of the parasites if studied within 4 hr after the inoculation. These spots were also seen in parasites released from cells bursting after 24 hr of infection, but then the parasites showed, in addition, fluorescence of the membrane in the anterior end and cytoplasmic structures resembling the paired organelles.

The appearance of immunofluorescence in the parasites in relation to the stage of infection is recorded in Table 8. The percentage of parasites showing immunofluorescence decreased shortly after the inoculation but increased again with the time of incubation, reaching the 75% level at 24 hr after infection of the cell cultures. With the *Toxoplasma* hyperimmune serum, almost all parasites demonstrated the characteristic staining of the pellicle independently of when they were observed during the infection.

DISCUSSION

Trophozoites of *T. gondii*, a protozoon recently classified as a member of the *Eimeriina*, a suborder of *Eucoccidia* (5, 7), multiplies by endodyogeny inside a living host cell. In a series of

papers (10, 11, 13–15), a factor has been studied which is synthesized by the parasites and which, by acting upon the wall of the host cells, facilitates the penetration into the host cells.

In the present paper, attempts were made to associate the PEF with a structural component of the trophozoites by means of the FA method. To achieve this, a specific antiserum had to be prepared by immunization with a satisfactorily purified antigen of PEF. As pointed out previously (11, 15) PEF seems to be a protein with an enzymatic activity. In accord with the proteineous nature of PEF, its effect on parasite-host cell interaction disappeared after treatment with phenol or trypsin. It could be precipitated with $(NH_4)_2SO_4$, and it migrated electrophoretically as a slightly acid protein. Like most enzymes, it was active in very small amounts. In fact, fractions obtained by Sephadex chromatography which were biologically highly active demonstrated spectrophotometrically minute, if any, amounts of protein. Therefore, it had to be anticipated that although preparations of PEF demonstrated a marked penetration-promoting activity, they contained little of PEF in substance. It was also found that, shortly after the addition of PEF to cultured cells, its penetration-promoting activity disappeared (13), indicating that PEF was used on contact with cells. Against this background, the difficulties encountered in evaluation of the purification of PEF and its seemingly low immunogenicity were explicable.

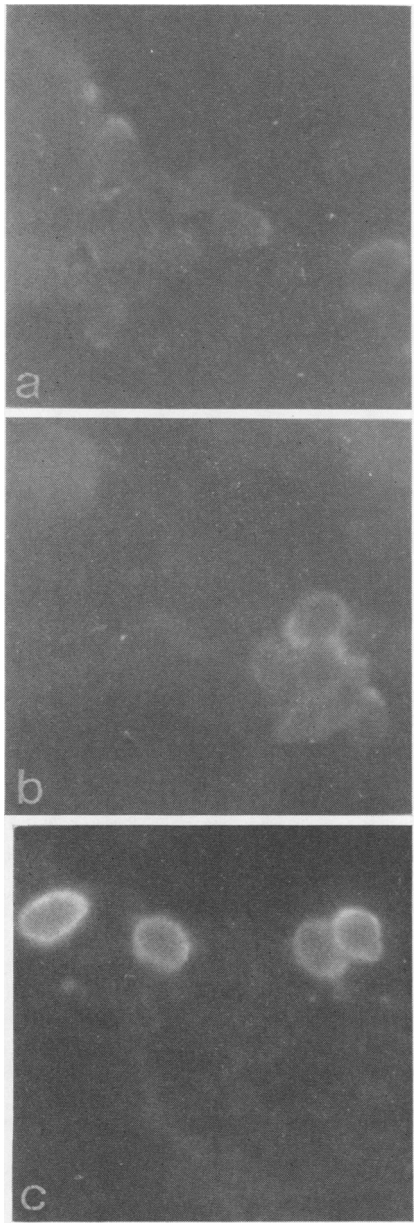


FIG. 7. *Immunofluorescence of Toxoplasma parasites. Toxoplasma-infected cell cultures, incubated for 24 hr, were fixed in acetone, incubated with 5× concentrated antiserum F (a, b) or with a Toxoplasma hyperimmune serum from rabbit (c) and, subsequently with a fluorescein isthiocyanate-conjugated antirabbit globulin diluted 1:2. × 1,500.*

By means of a combination of $(NH_4)_2SO_4$ precipitation at 30% saturation and a subsequent Sephadex G-200 gel filtration, two preparations of purified PEF were obtained. More than 99.9% of the extraneous protein, present in the original solutions of PEF which were obtained as extracts of *Toxoplasma* parasites, were removed by these procedures although the penetration-enhancing activity was increased about 2,500 times. Less than 0.001 μg of protein of these preparations was required to give demonstrable enhancement of the penetration.

The chromatography indicated that the penetration-enhancing activity was associated with at least two different components, one of which seemed to have a molecular weight between 70,000 and 150,000 and the other about 10 times larger. However, in both cases 37 C was found to be the optimal temperature for enhancement of the penetration, and no difference between the two components with regard to thermal inactivation or *p*H stability was observed. Moreover, the immune sera prepared by immunization with the two fractions of PEF showed cross-reactivity which indicates capacity to inhibit enhancement of penetration; tested in double diffusion in gel, the fractions themselves were found to contain only one and the same antigen. As, in addition, the same electrophoretic mobility was found for this precipitinogen and PEF, the results suggested that the difference in molecular weight between the components might be the result of aggregation of PEF.

TABLE 8. *Immunofluorescence of parasites at different stages of infection*[a]

Reactant	Immunofluorescence at[b]					
	0 hr	0.5 hr	1 hr	2 hr	4 hr	24 hr
Antiserum F	++	±	+	++	++	+++
Preimmune serum (F)	−	−	−	−	−	−
Toxoplasma hyperimmune serum	+++	+++	+++	+++	+++	+++
Hanks balanced salt solution	−	−	−	−	−	−

[a] Parasites of the inoculum and parasite-infected HeLa cells were incubated with antiserum F, preimmune serum, *Toxoplasma* hyperimmune serum, or Hanks balanced salt solution and, subsequently, with a fluorescein-conjugated antirabbit globulin.

[b] Immunofluorescence was recorded as (±) meaning fluorescence in less than 10%, (+) 10 to 25%, (++) 25 to 75%, and (+++) in more than 75% of the parasites.

When used in indirect immunofluorescence studies, an anti-PEF serum reacted with *Toxoplasma* trophozoites being stained according to a pattern clearly distinguishable from that obtained with a *Toxoplasma* hyperimmune serum. With the anti-PEF serum, small cytoplasmic structures of the anterior end of the parasites were stained. Particularly in parasites which had remained intracellular for 24 hr, the structures stained resembled the paired organelles or the convoluted tubes. It is in this context of interest that these structures have been suggested by Garnham et al. (5) to have a secretory function. Likewise, a secretory function effective during penetration has been attributed to structures located in the anterior end of malarian merozoites (1, 6, 8). We have previously reported (14) that lysosome-like bodies of *Toxoplasma* seemed associated with the capacity of penetration of the parasites. It is possible that the same structures studied in the latter report were studied with the FA technique used in the present report. In both cases, the results indicated that structures located in the anterior end of the parasites were associated with the penetration of the host cells and the synthesis of PEF.

ACKNOWLEDGMENTS

This study was supported by Public Health Service research grant AI 05074-08 from the National Institute of Allergy and Infectious Diseases.

I thank Mona Andersson and Agnete Svensson for their skillful technical assistance.

LITERATURE CITED

1. Aikawa, M. 1966. Fine structure of the erythrocytic stages of three avian malarial parasites, *Plasmodium fallax, P. lophurae* and *P. cathemerium*. Amer. J. Trop. Med. Hyg. **15**:449–471.
2. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillator. Anal. Biochem. **1**:279–285.
3. Field, A. K., A. A. Tytell, G. P. Lampson, and M. R. Hilleman. 1967. Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. Proc. Nat. Acad. Sci. U.S.A. **58**:1004–1018.
4. Frenkel, J. K., J. P. Dubey, and N. L. Miller. 1970. *Toxoplasma gondii* in cats: fecal stages identified as coccodian oocysts. Science **167**:893–896.
5. Garnham, P. C. C., J. Baker, and R. Bird. 1962. Fine structure of the cystic form of *Toxoplasma gondii*. Brit. Med. J. **1**:83–84.
6. Hepler, P., C. Huff, and H. Sprinz. 1966. The fine structure of the exoerythrocytic stage of *Plasmodium fallax*. J. Cell. Biol. **30**:333–358.
7. Hutchison, W. M., J. F. Dunachie, J. C. Siim, and K. Works. 1970. Coccodian like nature of *Toxoplasma gondii*. Brit. Med. J. **1**:142–144.
8. Ladda, R., M. Aikawa, and H. Sprinz. 1969. Penetration of erythrocytes by merozoites of mammalian and avian malarial parasites. J. Parasitol. **55**:633–644.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
10. Lycke, E., and E. Lund. 1964. A tissue culture method for titration of infectivity and determinaiton of growth rate of *Toxoplasma gondii*. Acta Pathol. Microbiol. Scand. **60**:209–233.
11. Lycke, E., and R. Norrby. 1966. Demonstration of a factor of *Toxoplasma gondii* enhancing the penetration of Toxoplasma parasites into cultured host cells. Brit. J. Exp. Pathol. **47**:248–256.
12. Lycke, E., R. Norrby, and J. Remington. 1968. Penetration enhancing factor extracted from *Toxoplasma gondii* which increases its virulence for mice. J. Bacteriol. **96**:785–788.
13. Norrby, R. 1970. Host cell penetration of *Toxoplasma gondii*. Infec. Immun. **2**:250–255.
14. Norrby, R., L. Lindholm, and E. Lycke. 1968. Lysosomes of *Toxoplasma gondii* and their possible relation to the host cell penetration of Toxoplasma. J. Bacteriol. **96**:916–919.
15. Norrby, R., and E. Lycke. 1967. Factors enhancing the host cell penetration of *Toxoplasma gondii*. J. Bacteriol. **99**:53–58.
16. Sabin, A. B., and H. A. Feldman. 1948. Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoon parasite (Toxoplasma). Science **108**:660–663.
17. Strannegård, Ö., and E. Lycke. 1966. Properdin and the antibody-effect on *Toxoplasma gondii*. Acta Pathol. Microbiol. Scand. **66**:227–238.
18. Wadsworth, C. 1957. A slide microtechnique for the analysis of immune precipitates in gel. Int. Arch. Allergy **10**:355–360.
19. Wadsworth, C. 1963. Comparative testing of a new photographic material for hand registration of immunoprecipitates. Int. Arch. Allergy **23**:103–114.
20. Wadsworth, C., and L. Å. Hanson. 1962. Comparative analysis of immune electrophoretic precipitates employing a modified electrophoretic technique. Int. Arch. Allergy **17**:165–177.