pathogenic African trypanosomes and suggested that at least some of the lesions observed are due to the action of a number of trypanosomes-derived factors. Factors like the trypanosome surface glycoproteins, anesthetics and other potentially toxic metabolites are produced by living trypanosomes, while other factors such as proteases, lipopolysaccharides and free fatty acids are released from dead or dying trypanosomes. Factors such as trypanosome-hemolysin, mitogens and hepatotoxins have also been described (Tizard et al., 1978a, 1978b).

Some of these protozoal extracts and products have been shown to induce protective immunity in rats to Plasmodium berghei infection (Grothaus and Kreier, 1980), to activate complement (Santoro et al., 1979), to inhibit lymphocyte proliferation induced by mitogens (Capron and Camus, 1979) and to have lymphocyte mitogenic activity (Greenwood and Vick, 1975). Lykins et al. (1971) found three soluble antigens in the serum of chickens infected with P. gallinaceum using the immunodiffusion test against an antisera obtained from chickens that survived the infection. In a series of publications, Soni and Cox (1974, 1975a,b,c) have shown that such antigens formed immune complexes and were responsible for glomerulonephritis, anemia and splenomegaly associated with acute P. gallinaceum infection of chickens.

Soluble antigens from Eimeria species have been demonstrated (as mentioned earlier) from extracts of infected tissues, oocysts, sporozoites, merozoites and second generation schizonts, using the agar immunodiffusion test (Rose, 1973). Some investigations were carried out to demonstrate toxic substances in homogenates of infected tissues or oocyst extracts (Burns, 1959; Sharma and Foster, 1964; Ryley, 1975). Unidentified toxic substances have been described by Burns (1959) from

homogenates of cecal contents, and scrapings taken from chickens infected with E. tenella and from homogenates of its oocysts. These extracts were toxic to rabbits when given intravenously or intraperitoneally. However, harmful effects were not produced when these were administered to chickens.

A substance, which was lethal to mice, was found in the peritoneal exudate of mice infected with Toxoplasma gondii and was called "Toxotoxin" by Weinman and Klatchko (1950). It was found that this material could not be extracted from the parasite, or produced in infected chick embryos or in infected tissue cultures (Weinman, 1952; Woodworth and Weinman, 1960; Nozik and O'Connor, 1969). According to Ryley (1975), "toxotoxin" is not a product of the parasite but rather a substance produced by host cells in response to infection, and its toxicity upon intravenous injection was attributed to its viscosity. Ryley (1975) believes that toxic substances that have been described in various coccidia have no bearing on the pathogenesis and they are little more than scientific curiosities.

Using anti-E. tenella serum in the immunofluorescent test, Davis et al. (1978) were able to demonstrate a diffuse fluorescence which was associated with villous and crypt epithelial cells of ceca of chickens infected with E. tenella. This diffuse fluorescence was attributed to the presence of soluble antigen, which was abolished by absorption with E. tenella antigen.

An acute inflammatory reaction characterized by severe hemorrhagic enteritis usually accompanies the development of second generation schizonts of E. necatrix. Soon after first generation merozoites penetrate crypt epithelial cells (as described in chapter two) these

cells migrate deep into the lamina propria and at the same time undergo morphological and functional changes such as the acquiring of phagocytic activity and leakiness of the plasma membrane. It was hypothesized that these developing parasites (or infected cells) produce metabolites which might damage host intestinal tissue either directly or indirectly by attracting leukocytes and creating an uncontrollable acute inflammatory reaction. Therefore experiments were undertaken to identify the possible presence of such diffusable parasite products and to isolate them in order to study their biological activities.

MATERIALS AND METHODS

1. Preparation of "soluble products"

For each preparation five chickens were infected with about 5 x 10^5 sporulated oocysts of E. necatrix per chicken. They were killed 4 days post infection, their intestines were removed, opened longitudinally and washed twice with cold 0.85% saline. The mucosa was scraped with a glass microscope slide and placed in a beaker kept in ice during the process of collection. The scrapings were weighed and suspended in cold PBS (pH 7.5), in the ratio of one milliliter per gram of tissue. The suspension was stirred in the cold (4.5°C) for two hours. In some instances, infected intestinal mucosa was suspended in EMEM* tissue culture medium containing 1.6 x 10^3 U penicillin and 640 ug Streptomycin and the mixture incubated for 4 hours at 37°C with continuous stirring. At the end of this period the mixture was centrifuged, first at 1500xg for 10 minutes (to remove the large pieces) and then at 16,000xg for 30 minutes at 4°C. The sediment was discarded

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* Gibco (Canada), Burlington, Ontario.

and the supernatant, designated "soluble products" was stored at -20°C until use. Supernatants prepared in the same manner from mucosal scrapings of uninfected chickens were used as controls.

2. Ammonium sulphate fractionation of "soluble products"

An equal volume of saturated ammonium sulphate was added dropwise with continuous stirring into the soluble products of second generation schizonts kept in a beaker surrounded by ice over a magnetic stirrer. The precipitate, which developed, was recovered by centrifugation at 16,000xg for 15 minutes. The sediment was redissolved in normal saline to half the original volume. The redissolved sediment and the supernatant were dialysed at 4°C against three changes of 3.5 litres of normal saline for 36 hours. The supernatant left after ammonium sulphate precipitation was concentrated to half the original volume by dialysis against polyvinylpyrolidone* (PVP). During the process of concentration a precipitate developed which was removed by centrifugation at 13,000 xg for 15 minutes.

3. Preparation of oocyst antigen(s)

Fifty milliliters of E. necatrix oocyst culture containing 5.5 x 10^5 sporulated oocysts/ml was used for this purpose. The culture was centrifuged at 1500 xg for 10 minutes to pellet the oocysts. To separate the oocysts from the debris, the pellet was suspended in sterile saturated sodium chloride solution and centrifuged at 1500 xg

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* Arthur H. Thomas Co., Philadelphia, Pennsylvania

for 10 minutes. The sediment was discarded and the supernatant containing the oocysts was dialysed at 4°C for 24 hours against a continuous flow of tap water. The oocysts were recovered by centrifugation at 1500 xg for 10 minutes, resuspended in 10 ml normal saline and counted in a hemocytometer chamber.

The oocysts were purified further with 6% sodium hypochloride solution (Javex*) according to the procedure described by Davis (1973) with some modification. The oocysts was pelleted by centrifugation, suspended in 7 ml of full strength Javex in a 50 ml plastic centrifuge tube** and overlayed with 10 ml distilled water. The gradient was then centrifuged at 650 xg for 10 minutes with the centrifuge brake off. The band of pure oocysts found at the sodium hypochloride/water interface was collected and washed extensively with normal saline. The sediment was resuspended in Javex and the same procedure repeated again to recover more oocysts.

The purified oocysts were suspended in 5 ml double distilled water in a plastic centrifuge tube. Glass beads*** were then added to the oocyst suspension (3.3 gm/0.8 ml) and shaken vigorously using the full speed of a Vortex mixer to break the oocysts, and sporocysts and lyse the liberated sporozoites. The disrupted oocyst contents were recovered by washing the glass beads with an additional 5 ml of distilled water. The preparation was centrifugated at 16,000xg for 30 minutes. The supernatant, regarded as oocyst antigen(s), was stored at -20°C until use.

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* Bristol-Myers Canada Ltd., Toronto
** Sorval, Norwalk, Connecticut, U.S.A.
*** Ferro, Cataphote Division, Jackson, Miss., U.S.A. Cat #353.5

4. Second generation merozoite purification

Merozoites were obtained from chickens killed 5.5 days post infection. Two chickens were infected with 1 x 10^4 sporulated oocysts of E. necatrix. At 5.5 days post infection they were killed and infected parts of the intestine cut into 2 cm long pieces with its contents. About 200 ml cold PBS was added to these and homogenized using a vertical homogenizer* at full speed for 5 minutes. The homogenized mucosa was centrifuged at 200 xg for 10 minutes at 4°C. Sediment was discarded and the supernatant filtered twice through two layers of gauze to remove fatty materials. After filtration the supernatant was centrifuged again at 1500xg for 10 minutes to recover second generation merozoites. To separate red blood cells and inflammatory cells from merozoites, the mixture was applied onto a discontinuous Percoll gradient. Stock isotonic percoll was prepared by mixing 9 parts Percoll** solution with 1 part of an 8.5% NaCl solution. The gradient consisted of 5 ml stock Percoll solution at the bottom overlayed with 15 ml of 7/3 parts stock Percoll plus 1/3 parts normal saline. Four, 50 ml tubes were used and each was overlayed with 5 ml of the crude merozoite suspension. Tubes were centrifuged at 16,000xg for 10 minutes at 4°C with the centrifuge brake off.

Pure merozoites were recovered from the interface between the two Percoll concentrations while leucocytes and other cells remained between the interface of the normal saline and the first layer of the Percoll.

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* Sorvall, Norwalk, Connecticut, U.S.A.
** Pharmacia Fine Chemicals, Uppsala, Sweden

5. Production of "anti-soluble products" sera in chickens

Four milliliters of "soluble Products" was emulsified with an equal volume of Freund's Complete Adjuvant. Four, 10-week-old chickens were each injected intamuscularly with 2 ml. Each dose was divided and injected into both sides of the legs. Three weeks later one milliliter of "soluble products" alone was injected into each chicken. Chickens were bled one week after the last injection.

6. Production of anti-oocyst serum

Six million sporulated oocysts purified as described earlier were suspended in 2 ml PBS and disrupted using glass beads and the Vortex mixer. Broken oocysts were recovered using 8 ml of PBS. Four milliliters of this suspension were mixed with an equal volume of Freund's Complete Adjuvant* and four, 10 week-old chickens were each inoculated i.m. with 2 ml. This dose was divided and injected into both sides of the legs and into the footpads. At the same time, 1 ml of the broken oocyst suspension was inoculated intravenously to each chicken. A second and third injection, each of 1.5×10^6 broken oocysts suspended in 2 ml PBS, were given to each chicken intramuscularly at 4 and 6 weeks respectively. One week later all chickens were bled from the heart.

7. Production of anti-*Eimeria necatrix* serum by oral infection

a) Chickens were immunized according to the schedules described by Rose and Long (1962) and Davis *et al*. (1978) with some modifications. Four chickens, 3 weeks of age were each infected orally with 2×10^4 sporulated oocysts of *Eimeria necatrix* suspended in 1 ml. They were

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* Difco Laboratories, Detroit 1, Michigan.

reinfected a second, third and fourth time with 2×10^4, 5×10^4 and 1×10^5 oocysts respectively at weekly intervals and bled 2 weeks after the last immunizing dose.

b) A second group of four chickens were immunized as described by Davis et al. (1978). They were each infected orally with 1×10^4 sporulated oocysts and injected at the same time with 2 ml of Freund's Complete Adjuvant intraperitontally. Sera were collected 2 weeks later.

8. Double immunodiffusion test (DID)

One percent ionagar* No. 2 in 4% sodium chloride (Davis et al., 1978) was used. Sodium azide in a concentration of 0.1% was added to prevent fungal and bacterial contamination. Nine milliliters of molten agar were poured on 2 x 3 inch slides placed on a leveled stage. After solidification the agar slides were stored in a moist chamber at 4°C until use. Two sets of wells were cut per slide using a commercial template.** Each set of wells consisted of one central well surrounded by six wells 4 mm in diameter and 7 mm apart. Reagents were added using disposable capillary pipettes. Slides were incubated in a moist chamber at room temperature and results were recorded daily for at least 3 days post application of antisera and antigens.

9. DID using infected intestinal tissue

The method described by Taylor (1978) was used with some modification. Chickens were infected with 3×10^5 sporulated oocysts of

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* Oxoid Limited, London, S.E. England.
** Gelman Instrument Company, Ann Arbor, Michigan.

E. necatrix and killed 4 days post infection. Heavily infected areas of the intestine (jejunum) were frozen at -70°C to facilitate the cutting of small rings of intestine, 2-3 mm thick. Wells were cut in the agar gel diffusion medium as described earlier, and the intestinal tissue rings placed in these wells so that any diffusible antigen present could interact against anti-oocyst serum placed in an adjacent well. A few drops of PBS pH 7.4 was added to these intestinal rings to facilitate the transport of the soluble antigen. Rings of normal intestinal tissue were prepared in the same manner and used as controls.

10. Staining of immunodiffusion slides

When precipitation lines were clear, slides were soaked for approximately 3 days in 3-6 changes of normal saline containing 0.1% sodium azide to wash out the unprecipitated proteins. To remove salts, the slides were rinsed once and then soaked for at least 10 hours in distilled water. The slides were dried after overlaying the agar surface with moist filter paper. Care was taken not to trap air bubbles between the agar and the filter paper. The slides were dried at 40°C overnight. Dried slides were stained with Amido black* and decolorized with two decolorizers to remove the background staining (Appendix VI).

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* Fisher Scientific Co. Limited, Don Mills, Ontario.

11. Fractionation of "soluble products"

i. Separation of Sephadex G-200

Sephadex* G-200 was swollen by boiling for 5 hours in PBS pH 7.4.

"Soluble products" prepared using EMEM was fractionated on Sephadex G-200.

The "soluble products" were dialysed overnight against the equilibrating buffer (PBS pH 7.4). Twenty milliliters were applied on a 1.5 x 35 cm column packed with Sephadex G-50 coarse. The first peak (monitored by a UV spectrophotometer at 280 nm) was collected and concentrated to half the original volume by dialysis against PVP. Ten milliliters of this concentrate was applied on an 80 cm column packed with Sephadex-G200. The flow rate was about 36 ml/hour. Using a fraction collector, fractions of 4 ml/tube were collected.

RESULTS

1. Detection of "soluble products"

Attempts to detect diffusable parasite antigens in the supernatant of infected tissue suspensions using DID test with sera of chickens infected orally were unsuccessful. Accordingly, it was decided to prepare antisera against E. necatrix oocyst antigen(s) in chickens by parenteral immunization to ascertain the presence of parasite antigens in preparations of "soluble products".

Antigenic activity was detected in DID tests using the antisera prepared in chickens to whole oocyst antigens and the antisera prepared

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* Pharmacia Fine Chemicals, Uppsala, Sweden

from orally infected chickens that received the i.p. adjuvant. A line of complete identity was obtained using these two antisera against "soluble products" prepared from cells harbouring second generation schizonts (Fig. 4-1). Using whole oocyst antigens, two lines were detected against anti-oocyst serum; one of them in complete identity with the line developed with antigen prepared from second generation merozoites and the other with that of "soluble products" of second generation schizonts (Fig. 4-2). No lines of precipitation were developed between supernatant of normal mucosal scrapings and the antisera used in the DID test.

2. DID using infected intestinal tissue

Infected intestinal tissue rings obtained at 4 days post infection were used as a source of second generation "soluble products" in DID tests. Precipitation lines were obtained against anti-oocyst serum and the antiserum prepared by oral immunization. These lines were in complete identity with those produced by the "soluble products" of second generation schizonts (Fig. 4-3).

No line of precipitation was developed between uninfected intestinal tissue rings and the antisera used (Fig. 4-3).

3. Ammonium sulphate precipitation of "soluble antigen"

When 50% saturated ammonium sulphate was added to the "soluble products", a heavy precipitate was formed. Both the supernatant and precipitate were tested for antigenic activity after dialysis and concentration. Using DID test, the antigenic activity was only detected in the redissolved sediment but not in the supernatant.

4. Sephadex G-200 Fractionation

The fractionation of "soluble products" on Sephadex G-200 column revealed three peaks when monitored at 280 nm (Fig. 4-4). Fraction nos. 14-25 were pooled and labelled P1, fraction nos. 26-55 labelled P2 and fraction nos. 56-80 were labelled P3. When these three peaks were tested, only P2 demonstrated a line of precipitation against anti-oocyst and anti-soluble product sera. No lines of precipitation were detected with P1 and P3 when tested against the same two antisera.