buffered saline (PBS) with the aid of a squeeze bottle. Soon after washing, the pieces were stretched by pinning them on a flat piece of rubber, immersed in fixative (2% glutaraldehyde and 2.5% paraformaldehyde in phosphate buffer pH 7.2 prepared according to Karnovsky, 1965) and kept at 4°C until trimmed. Trimming was done longitudinally into small rectangular pieces (2 mm x 3-4 mm) to facilitate their orientation during embedding.

Trimmed tissues were washed three times for 10 minutes each in phosphate buffered saline and post fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 to 1.5 hours. Osmium tetroxide was removed by suction and the tissues were washed three times with bidistilled water and stained in a 2% aqueous solution of uranyl acetate for two hours in the dark.

ii. Dehydration

After staining with uranyl acetate, the tissues were washed three times with PBS before dehydration. They were dehydrated in increasing concentrations of acetone in distilled water. Tissues were dehydrated once in 50%, 70%, 90% and twice in 100% acetone, each time for 15 minutes. Pieces of calcium chloride were added to the 100% acetone to remove any moisture.

iii. Embedding

Spurr*, which is an epoxy resin, was used for embedding tissues because of its low viscosity. To prepare the standard embedding medium (Hard spurr), four different components were weighed and mixed, according to the instruction manual, as follows:

---

* Polysciences Inc., Paul Valley Industrial Park, Warrington, PA 18976

Vingylcyclohexene dioxide (VCD)	10 g
Diglycidyl ether of polypropylene glycol (DER)	6 g
Noneyl succinic anhydride (NSA)	26 g
Dimethylaminoethanol (DMAE)	0.4 g

These components were thoroughly mixed with a magnetic stirrer before use.

To infiltrate tissues, 25, 50 and 75% spurr in 100% acetone was prepared and used as follows:

1 - 25 parts spurr and 75 parts 100% acetone for one hour

2 - 50 parts spurr and 50 parts 100% acetone for one hour

3 - 75 parts spurr and 25 parts 100% acetone for one hour

Then tissues were left overnight in 100% spurr. A small amount of spurr was distributed in a flat embedding capsule, and small labels to identify individual tissues were placed in each well. Pieces of tissue were then transferred to these wells and oriented as required and placed in a 70°C oven for 16-24 hours.

iv. Sectioning of embedded tissue

Blocks were trimmed before they were mounted on a Reichert ultramicrotome* for the preparation of thick and thin sections. Thick, 1 micron sections were cut, and transferred onto microscope slides, flattened by placing them on a hot plate and then stained with a 1% aqueous solution of methylene blue.

Golden-colored (90-100 nm) sections placed on 200 or 300 mesh copper grids were stained with an aqueous solution of 2% uranyl acetate and 0.3% lead citrate as follows:

---

* Sargent-Welch Scientific Co., 285 Garyray Drive, Weston, Toronto, Ont.

Uranyl acetate solution was drawn from the middle of the bottle to avoid dust on the top and crystals on the bottom of the solution. A few single drops (depending on the number of grids to be stained) were placed on wax in a petri dish. One grid, with the surface containing the section facing down, was placed on each drop of uranyl acetate and left for 15 to 20 minutes at room temperature. At the end of this period, grids were removed one by one and washed by dipping rapidly in and out of double distilled water. This was done 10 times in each of 3 beakers containing double distilled water.

The same procedures were repeated using lead citrate. Drops of lead citrate were placed in a petri dish containing pellets of NaOH in a center hole in the wax. Grids were stained for 10 to 15 minutes and washed as before except that 4 drops of 1 N NaOH were added to the first beaker to help prevent lead citrate from precipitating.

Stained sections were examined with a Hitachi HS7 electron microscope at 75 kv.

Hematological Procedures

1. New methylene blue stain

New methylene blue stain was used to facilitate total leucocyte counts. A 1% stock solution was prepared in distilled water and kept at room temperature. The working dilution was prepared by mixing 1 ml of the stock solution with 30 ml of PBS pH 7.4 containing 3% formalin. The working solution was distributed in 1 ml volumes into small screw capped bottles containing two small glass beads of about 2 mm diameter to facilitate mixing.

2. Total and differential leucocyte counts

Blood samples were obtained by puncturing the wing vein with a 25 gauge sterile needle. Blood was drawn into a 10 microliter capillary tube, transferred into a small screw capped bottle containing 1 ml of the working dilution of new methylene blue and mixed by shaking the bottle. Total leucocyte counts were made in a white blood cell counting chamber following standard laboratory procedures.

Blood films for differential leucocyte counts were prepared at the same time, air dried and stained with Wright's stain using an automatic staining machine. Differential counts were made on two hundred white blood cells per slide. No attempts were made to count platelets in stained smears.

Statistical Method

The student t-test was used to test the level of significance wherever statistical analysis was performed.

CHAPTER TWO

PATHOLOGICAL CHANGES ASSOCIATED WITH *EIMERIA NECATRIX* INFECTION - ROLE OF LEUCOCYTES IN TISSUE DAMAGE.

INTRODUCTION

E. necatrix infection is usually accompanied by an acute inflammatory reaction in the chicken intestine.

In mammals, leucocytes that infiltrate the tissue in the early stages of the typical acute inflammatory response are neutrophilic polymorphonuclear leucocytes (PMN). Later in the response the prevailing cells are blood-derived mononuclear cells and macrophages. Physiological function of PMN and macrophages is ingesting and digesting particulate matter such as bacteria and damaged cells. Eosinophils and basophils may infiltrate in large numbers under special circumstances as part of the inflammatory response (Bainton 1980).

In chickens, several workers have documented the infiltration of heterophils, mononuclear cells and basophils during the early stages of an irritant induced acute inflammatory reaction (Carlson and Allen, 1970; Jortner and Adams, 1971; Nair, 1973). Carlson and Allen (1970) reported that the sequence of the appearance of the various types of leucocytes and the time intervals was consistent regardless of the type of irritant used. By half an hour, there was infiltration of heterophils, mononuclear cells and basophils from the dilated blood vessels into the surrounding tissue. However, the early lymphocytic infiltration (12 hours post injection) is the most constant and characteristic observation in acute inflammation of chickens and that

deviates most from the mammalian reaction (Carlson and Allen, 1970). According to Nair (1973) the life span of the heterophil is short and this, in addition to the persistence of infiltration of mononuclear cells, accounts for the conversion of the heterophil rich exudate to one with mononuclear predominance.

Inflammation may also be the result of certain immunological reactions. Chand, Carlson and Eyre (1976) found that the main cells which infiltrated the skin lesion in chickens as a result of a passive cutaneous anaphylaxis reaction were heterophils, mononuclear cells and some basophils. It was suggested that circulating basophils may be responsible for the mediation of early acute inflammatory reaction of immunologic origin. Rose and Bradley (1977) studied the local cellular response in delayed hypersensitivity reactions in sensitized chicken, turkey and quail at 48 hours after challenge observed a moderate to marked perivascular infiltration of mononuclear cells and a small but more diffuse accumulation of heterophils at the site of injection.

It was possible a long time ago to establish that leucocytes, especially PMN, were responsible for certain forms of tissue injury. The Arthus reaction, which is an acute, necrotizing inflammatory lesion of venules, was the first antigen- antibody-induced lesion found to be dependent on PMN's (Cochrane, 1977). The activation of complement by immune complexes around venules resulted in accumulation of neutrophils at the site and injury to the vessel walls leading to edema and hemorrhage. Specific removal of neutrophils prior to eliciting the reaction has been shown to lead to striking inhibition of the injury in several species (Humphrey, 1955a,b; Cochrane, Weigle and Dixon, 1959). PMN's were also found to be important in serum sickness of rabbits and

acute nephrotoxic nephritis in rats and rabbits (Cochrane, 1977). According to Cochrane (1977) any local source of antigen will initiate these immunological lesions, once antibody is formed. When the immune complex is capable of activating complement, PMN's will accumulate leading to release of biologically active constituents of these cells and, depending on the site of deposition, it may lead to glomerulonephritis, synovitis, arteritis and vasculitis. Thus it is now appreciated that PMN's are not merely innocent bystanders at an inflamatory site, but play an active role in the mediation of tissue injury. Central to this role are the inflammatory substances contained within the cytoplasmic granules of these cells, the lysosomes (Goldstein, 1974).

Extensive tissue damage usually occurs in the intestine of chickens infected with E. necatrix. Uninfected tissue in the vicinity of cells harbouring second generation schizonts seems to be particularly affected. Heterophil infiltration has been described by Stockdale and Fernando (1975) as a prominent feature at the beginning of second generation schizogony. The time at which tissue changes and lesions first appear in the intestine coincides with this massive infiltration of leucocytes especially heterophils. Experiments were therefore undertaken to study (1) the leucocyte response in relation to the development of different endogenous stages of E. necatrix and (2) the macroscopic, light microscopic and ultrastructural changes associated with the acute inflammatory response during the development of second generation schizonts.

MATERIALS AND METHODS

1. Macroscopic, light and electron microscopic changes.

For ultrastructural studies, chickens inoculated with $1x10^6$ oocysts were killed at 65, 67, 69, 70 and 72 hours post infection and those inoculated with $1x10^5$ oocysts killed at 120 hours post infection. Three chickens were killed at each time post infection and intestinal tissues processed as described in Chapter 1 under electron microscopy.

Lesions in chickens used for ultrastructural studies were also assessed in terms of sequence of development and changes at the macroscopic and light microscopic levels.

2. Peripheral leucocyte response

Sixteen chickens were assigned to four groups of four chickens each. Chickens in groups 1, 2 and 3 were each inoculated with $1x10^3$, $5x10^3$ and $1x10^4$ oocysts respectively. Chickens in group 4 served as uninoculated controls. Control chickens were kept in a clean room under conditions similar to the inoculated groups. Chickens in all groups were bled from the wing vein before commencing the experiment and then every 24 hours up to day 14 post infection. Total and differential leucocyte counts were performed as described in Chapter 1. Total daily oocyst production per group was estimated as detailed in Chapter 1.

Feces was collected every 24 hours from day 6 to day 13 post infection.

3. Isolation of heterophils from peritoneal exudate

i. Experimental peritonitis

Massive infiltration of leucocytes is known to follow the injection of an irritant into the peritoneal cavity of chickens. Heterophils represents the majority of these cells during the first day after injection. Therefore peritoneal exudate was used as a source of heterophils. For each isolation five chickens (10-15 weeks old) were injected intraperitoneally (i.p.) with 15-20 ml of 10% sterile proteose peptone in normal saline as described by Trifonova et al. (1977). Eighteen hours later a second i.p. injection of 10 ml of proteose peptone was given. Three hours after the second injection, the chickens were sacrificed, the peritoneal cavity was opened and exudate collection in cold Seligman's Balanced Salt Solution (SBSS) (Appendix IV). Cells were distributed into four, 50 ml plastic centrifuge tubes and washed twice in SBSS by centrifugation at 650 xg for 10 minutes each. Cells in each tube were resuspended in the same buffer with the aid of a Vortex mixer and applied on a discontinuous Percoll gradient.

ii. Percoll gradient

Stock Percoll solution was prepared as 9 parts Percoll plus 1 part 8.5% NaCl solution. The discontinuous gradient was prepared by diluting stock Percoll solution in SBSS containing 10 units of heparin/ml. Five milliliters of stock Percoll was placed at the bottom of each 50 ml tube followed by 6 ml of 70% stock (7 parts stock Percoll, 3 parts buffer) followed by 8 ml of 40% stock (4 parts stock Percoll plus 6 parts buffer). Peritoneal exudate cells, suspended in 4 ml buffer, was applied gently to the top of each tube (total 4 tubes) and centrifuged at 16,000 xg for 10 minutes with the break off.

4. Production of anti-heterophils serum in rabbits

Pure heterophils were collected from Percoll gradients (see Fig. 2-36 and results), washed twice in SBSS buffer, counted and injected into rabbits as follows. Six New Zealand white rabbits were injected intravenously with purified heterophils. Each rabbit received four injections, totalling 3.76×10^8 heterophils, given at weekly intervals. Rabbits were bled 10 days after the last injection.

Another group of four rabbits were injected subcutaneously at four different sites with a total of 2.8×10^8 heterophils per rabbit suspended in 2 ml of SBSS and emulsfied with an equal volume of Freund's Complete Adjuvant. A second s.c injection of 2.4×10^8 heterophils suspended in 4 ml of the collecting buffer was given per rabbit one month later at 3-4 different sites. The rabbits were injected with a third and fourth injection of 2.0×10^8 and 1.8×10^8 heterophils respectively in Freund's Incomplete Adjuvant at montly intervals. Rabbits were bled 10 days after the last injection.

5. Absorption of rabbit anti-heterophil serum

i. Preparation of chicken red blood cells (RBC's)

Ten chickens, 14 weeks old, were bled from the heart. Blood was collected in an equal volume of Alsever's Solution (Appendix V). Collected blood was left overnight in 50 ml centrifuge tubes at 4°C. The supernatant was discarded and normal saline was added to make up the original volumes and the tubes were centrifuged at 1500 xg for 10 minutes. The buffer coat was removed completely by suction, and the remaining RBC's were washed twice in normal saline by centrifugation at 650 xg for 10 minutes each. Packed cell volume was determined and the cells stored at 4°C until used for absorption.