ii. Preparation of heterophil free peritoneal exudate cells

Peritoneal exudate was obtained as described earlier by i.p. injection of proteose peptone. Cells were separated on the discontinuous Percoll gradient that was used for heterophil isolation. Leucocytes other than heterophils were collected (see Fig. 2-36 and results) and separated on a second gradient similar to the first, to reduce as much as possible, heterophil contamination. Cells were pelleted by centrifugation at 2000 xg for 5 minues and used for absorption of heterophil serum.

iii. Preparation of lymphocytes from thymus and bursa

The lymphocytes of thymus and bursa were prepared as described by Pace, Barger, Dawe and Ragland (1978). The thymuses and bursas of four chickens were used. Thymuses were cleaned from adhering fatty tissues and washed in cold SBSS. Thymus and bursal cells were isolated by gently passing the tissues through a sterile stainless steel mesh, with the aid of a small rubber stopper, into a petri dish containing SBSS. The cells obtained from both organs were mixed together suspended in SBSS, washed three times in the same solution, packed by centrifugation at 1500 xg for 10 minutes and stored at 4°C for a few minutes before being used for absorption.

iv. Preparation of polymerized chicken plasma proteins

Three chickens (15 weeks of age) were bled from the heart. Blood was collected in equal volumes of SBSS. About 70 ml of diluted plasma was collected and used for polymerization. One milliliter of 1.0 M acetate buffer pH 5.0 (Appendix VI) was added to every 10 ml of diluted plasma. Three ml of 8.5% aqueous gluteraldehyde solution per 10 ml of diluted plasma was added dropwise. The pH of the mixture was

adjusted to 5.0 with acetate buffer during the addition of gluteraldehyde. The mixture was left at room temperature until polymerization was completed (about three hours). The polymeried plasma was washed twice in distilled water and twice in 0.1 M glycine HCl buffer pH 2.8 (Appendix VI) and twice in 0.2 M PBS pH 7.4 (Appendix III). Finally, the polymerized plasma proteins were centrifuged at 4000 xg, supernatant was discarded and the pellet stored in the same centrifuge tube at -20°C until use.

v. Preparation of acetone treated tissue

Livers, bursas and thymuses of the 10 chickens used for the preparation of leucocyte free RBC's were homogenized together without addition of diluent. The homogenized tissues were suspended in 800 ml of 100% acetone (about four times tissue volume) and shaken manually for about 5 minutes. The mixture was centrifuged at 1500 xg for 15 minutes, the acetone layer was discarded and the tissue homogenate washed three times in PBS before use for serum absorption.

vi. Anti-heterophil serum absorption

Sera obtained from the first immunization schedule were pooled and mixed with acetone treated tissue in the ratio of 1 gm wet tissue to every 2 ml serum. Then 1 ml packed chicken red cells was addd to every 7 ml of the serum. The mixture was left overnight in the cold room (4°C) on a rotary shaker set at 120 RPM/minute. The mixture was centrifuged at 1500 xg for 15 minutes and the supernatant (anti-serum) centrifuged at 16,000 xg to remove fine tissue pieces. This was followed by absorption with heterophil-free peritoneal exudate cells (mainy macrophages). One hundred milliliters was mixed with packed cells to give a final concentration of 4.4×10^7 cells/ml and

incubated at room temperature with continuous shaking for 1 hour. The absorbed serum was recovered by centrifugation at 16,000 xg for 30 minutes and stored at -20 until use.

Anti-heterophil sera obtained from the second immunization schedule were pooled and heat inactivated at 56°C for 30 minutes. The pooled bursa and thymus lymphocyte suspension obtained from 4 chickens was mixed with 40 ml of the anti-heterophil serum with continuous mixing on a rotary shaker for 90 minutes at room temperature (Pace et al., 1978) and for an additional 2 hours at 4°C. Lymphocytes were removed by centrifugation at 1500 xg for 5 minutes and a subsequent centrifugation at 16,000 xg for 1 hour at 4°C. Ten milliliters of packed chicken RBC's were then added to the anti-heterophil serum and absorbed overnight at 4°C. The next morning the anti-serum was recovered by centrifugation and reabsorbed with 5 gm of polymerized chicken plasma for 8 hours at 4°C with continuous mixing. The anti-serum was again recovered by centrifugation at 16,000 xg for one hour and stored at -20 until use.

6. Preparation of tissue antigens for double immunodiffusion test (DID)

The brain, liver, breast muscle, bursa and thymus were collected from two chickens. Tissues were washed and rinsed carefully with normal saline and cleaned of clotted blood and fatty tissue. The livers were treated differently. They were cut into small pieces and washed three times with normal saline, then soaked in normal saline and left overnight at 4°C. Each tissue was homogenized separately, frozen and thawed 5 times, and centrifuged at 16,000 xg for 10 minutes. The supernatant after centrifugation was used in the DID against anti-heterophil serum. Pure heterophils, and heterophil free peritoneal

exudate cell antigens, were prepared by freezing and thawing 5 times and then by homogenizing them further using a glass homogenizer. Cell fragments were centrifuged at 16,000 xg for 10 minutes and the supernatant was used in the DID test.

7. Titration of anti-heterophil serum

i. Cytotoxicity assay

This was done as described by Simpson and Ross (1971). Doubling dilutions from 1/4 to 1/1024 of duplicates of absorbed anti-heterophil serum and one set of heat inactivated normal rabbit serum were prepared in RPMI tissue culture medium. Two milliliters of live hterophils ($5.3x10^7$/ml) were added to 48 ml of RPMI medium. To every serum dilution (0.4 ml) equal volumes of the heterophil suspension and 10% normal fresh rabbit serum were added and the tubes were incubated at 37°C for 30 minutes. One milliliter of 0.01% trypan blue in normal saline was added to very tube and the proportion of live heterophils was estimated in each tube using a white blood cell counting chamber.

ii. Agglutination assay

This was performed essentially as described by Simpson and Ross (1971). Doubling dilutions from 1/10 to 1/1280 of anti-heterophil and normal rabbit serum in 0.5 ml SBSS or PBS (pH 7.4) were prepared in small siliconized test tubes, and 2.5 x 10^6 heterophils in 0.5 ml was added to every tube. Tubes were incubated at room temperature for 1.5 hours before reading. Agglutination was assessed by phase-contrast microscopy at 250 times magnification after briefly shaking each tube and placing a drop of the cell suspension on a slide without a cover slip. The end point was taken as the highest dilution of anti-

heterophil serum containing distinct clumps of cells, approximately 10 or more per clump, when compared to control tubes of normal rabbit serum which usually contained smaller aggregates.

8. Experimental design for *in vivo* use of anti-heterophil serum

The first three experiments were performed using the absorbed anti-heterophil serum obtained from the first immunization as follows:-

Experiment 1

Eight chickens were divided into two unequal groups. The five chickens in group 1 were injected i.p. with 3 ml of anti-heterophil serum and the three chickens in group 2 injectd with 3 ml of normal rabbit serum. Total and differential leucocyte counts were performed one day before injection and at 1/2, 1, 6, 12, 24, 48, 72, 96 and 120 hours post injection.

Experiment 2

Ten chickens were divided into two groups of equal numbers. Chickens in group 1 and 2 were each injectd intravenously with 3 ml of anti-heterophil serum and normal rabbit serum respectively. All chickens were bled at 0, 1, 3, 6, 12, 24, 48 and 72 hours post injection for leucocyte counts.

Experiment 3

This experiment was the same as experiment two except that chickens in group 1 were injected with 2 ml anti-heterophil serum plus 1 ml of

fresh normal rabbit serum and chickens in group 2 with 2 ml of heat inactivated normal rabbit serum plus 1 ml of fresh normal rabbit serum.

The following experiment was done using the anti-heterophil serum obtained from the second schedule of immunization.

Experiment 4

Six 25-day-old chickens were divided into two equal groups. Chickens in group 1 were each injected with 2 ml of anti-heterophil serum plus 0.5 ml fresh guinea pig serum. One milliliter of this mixture was injected intraperitoneally and the rest injected intramuscularly into both legs. Chickens in group 2 were injected in the same way but with normal rabbit serum instead of anti-heterophil serum.

RESULTS

1. Lesions associated with the development of first and second generation schizonts.

No macroscopic or microscopic lesions were detected during the development of first generation schizonts (Fig. 2-1). No macroscopic lesions were observed during the early stages (up to 72 hours post infection) in the development of second generation schizonts. However, histologically, extensive heterophil infiltration was seen as early as 67 hours post infection (Figs. 2-1, 2-2, 2-3). Heterophils could be seen around, between epithelial cells and in the lumen of infected crypts (Figs. 2-3, 2-4). These changes were seen very soon after first generation merozoites were observed in crypt epithelial cells (65-70 hours post infection). As the parasite developed, increasing numbers of

heterophils were seen in the crypt lumen with very few around the parasitized cells (Fig. 2-5). Inside the lumen of infected crypts, heterophils in different stages of degeneration were seen (Fig. 2-5). Although blood vessels were seen close to the crypts, the origin of heterophil infiltration was not clear (Figs. 2-7, 2-8).

In Giemsa stained histological sections a few basophils were seen but unlike heterophils, they did not appear to be close to infected cells (Fig. 2-6).

Petechial hemorrhages were first detected later than 72 hours post infection. They increased in intensity during the infection and were very prominent even before the maturation and rupture of second generation schizonts.

Some chickens died at 4.5 days post infection. In such cases, the intestine contained a clotted blood cast which filled the lumen and was easily detached from a clear, smooth mucosa. Hemorrhages seemed to originate from blood vessels in the lamina propria.

Electron microscopic examination of the development of early second generation schizonts (70-72 hours post infection) clearly showed that early second generation trophozoites were found in crypt epithelial cells (Figs. 2-3, 2-9, 2-11). Even goblet cells were found to be infected (Fig. 2-4). Soon afterwards a two layered crypt with uninfected cells close to the lumen were seen (Figs. 2-9, 2-10). Infected crypt epithelial cells stained paler than uninfected cells. Desmosomes were seen between infected and uninfected crypt epithelial cells at this stage (Fig. 2-11). Infected cells at the periphery of the crypt appear to migrate through the basal lamina of the crypt towards the lamina propria (Figs. 2-12, 2-13).

Once in the lamina propria infected cells enlarge and are phagocytic (Fig. 2-14). Uninfected epithelial cells that remain to form the crypt appear flattened and some infected cells harboring mature schizonts may break out into the intestinal lumen through these areas. Infected cells harboring mature second generation schizonts are often seen at different levels of the villous lamina propria. Thus some infected cells may leave the mucosa through the tips of the villi. Electron microscopic examination of infected cells at 5 days post infection demonstrated many cytoplasmic projections and vesicle formations which gave the plasma membrane a very rough appearance (Fig. 2-15). Even when second generation merozoites were fully mature, the organelles of infected cells appeared ultrastructurally normal (Fig. 2-14).

2. Peripheral leucocyte response

Mean values of total peripheral leucocytes obtained over a period of 14 days after inoculation of $1x10^3$, $5x10^3$ and $1x10^4$ oocysts, are shown in Figs. 2-16, 2-17, 2-18, respectively. Mean values of peripheral leucocytes in coccidia-free chickens and the daily fecal oocyst output of infected chickens are also shown in the same figures. Peripheral blood leucocytes increased in the infected groups by day 9 post infection and returned to near normal values by day 14. This increase over the controls was statistically significant ($P \leq 0.05$) at day 10 in the group inoculated with $1x10^3$ oocysts, at days 10, 11, 12, 14 in the group inoculated with $5x10^3$ oocysts, and at days 9, 10, 11, 12, 13 in the group inoculated with 10,000 oocysts. The increase in the number of leucocyts was related to the number of oocysts inoculated.

The higher the inoculation dose the higher were the peripheral leucocyte counts (Figs. 2-16, 2-17, 2-18).

In the two groups receiving $1x10^3$ and $10x10^3$ oocysts, the first oocyst peak was higher than the second one (Figs. 2-16, 2-18). On the other hand, the first peak was not prominent in the group that received $5x10^3$ oocysts (Fig. 2-17). Peripheral blood leucocyte counts in the infected groups were generally lower than the controls during the first week post infection (Figs. 2-16, 2-17, 2-18).

Mean values of differential leucocyte counts are presented in Figs. 2-19, 2-20, 2-21, 2-22, 2-25). Heterophils increased during the infection showing two peaks, the first peak between day 5 and 6 and the second between day 9 and 10 post infection (Fig. 2-19). In all infected groups the first peak was the lower of the two. The number of small lymphocytes in infected groups was generally lower than in the controls during the first 8 days post infection (Fig. 2-20). By day 9 post infection, values returned to near normal or were significantly ($P \leq 0.05$) higher especially in the two groups inoculated with $5x10^3$ and $10x10^3$ oocysts. Lymphopenia was most evident during the period between day 5 and 9 in the three infected groups (Fig. 2-20). Increase in mean values of large mononuclear cells (LMNC) was seen by day 5 in the group that received the $5x10^3$ oocysts (Fig. 2-21). All infected groups showed higher numbers of LMNC than the controls between day 6 and 13 post infection. Three peaks of LMNC can be seen in Figure 2-21. These were more evident in the group which received $10x10^3$ oocysts. Chickens inoculated with $5x10^3$ oocysts showed the most marked increase in LMNC of all groups. Mean values of eosinophils started to increase after day 9 reaching a maximum at days 12 and 13 post infection

(Figs. 2-22, 2-23, 2-24). The increase in eosinophil numbers was related to the dose of oocysts inoculated (Fig. 2-22). The higher the oocyst dose the higher were the eosinophil numbers (Fig. 2-22). There were no dramatic changes in basophil numbers though they were generally lower in the infected groups during the period between day 2 and 8 post infection. Thereafter their numbers were comparable to the uninfected group (Fig. 2-25).

3. Isolation of heterophils

Early attempts to isolate large numbers of heterophils from chicken blood using the Percoll gradient method were not satisfactory. Heterophil numbers were low and insufficient for rabbit immunization. In addition, the very poor band of heterophils obtained after Percoll gradient centrifugation was very close to the red blood cell band and resulted in heavy contamination. Therefore, the peritoneal cellular exudate that formed after proteose peptone injection was used instead of blood for the isolation of heterophils. This method results in (1) low red blood cell contamination and (2) very high recovery of heterophils (between 70-80%) compared to other leucocytes.

After centrifugation of the Percoll gradients, two heterophil rich bands were detected just above the faint band of red blood cells. Macrophages and mononuclear cells were found at the top of the gradient (Fig. 2-36). Heterophil bands were easily collected and contamination with other cells was minimal. In some preparations, close to 100% purity could be achieved. An average of 2.0×10^8 heterophils per chicken was obtained in most preparations.