# CELLULAR ASPECTS OF THE RESISTANCE OF CHICKENS TO *EIMERIA TENELLA* INFECTIONS

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ABSTRACT

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The strong species specific immunity of chickens to reinfection by *Eimeria tenella* has been shown to be active against the parasite before merogony of first generation schizonts. At this time in the life cycle of the parasite, macrophages have been demonstrated transporting sporozoites in the gut lamina propria apparently unharmed by the cell. They are released from the macrophages and initiate infections in the epithelial cells of the deep glands of the ceca of susceptible chickens.

Peritoneal exudates were stimulated by injections of Hanks' balanced salt solution (BSS) in normal chickens and in chickens immunized against *Eimeria tenella* by three weekly graded oral inoculations of sporulated oocysts. In some of the experiments, the cells of the exudate were treated with normal serum or serum derived from chickens resistant to *E. tenella* by intraperitoneal injections of the serum during the development of the exudate. Forty-eight hours after the injection of BSS, sporozoites of *E. tenella* were injected

intraperitoneally into the chickens. After 15 or 30 minutes incubation, the chickens were killed and the exudates were harvested. The cells in a sample of the exudate were allowed to settle on coverslips for 15 minutes and the preparation was examined microscopically. The number of cells containing sporozoites out of a total of 500 cells was obtained.

Only macrophages and degranulated heterophil leucocytes were observed to contain sporozoites. There was no significant difference between the percent of infected immune macrophages and the percent of infected normal macrophages after 15 minutes. Significantly fewer immune macrophages treated with immune serum contained sporozoites than untreated normal or immune cells, normal macrophages treated with either serum, or immune macrophages treated with normal serum. The sporozoites in macrophages from the experimental groups with significantly lower percents of infected cells were visibly distorted and it is hypothesized that the fewer observed infected cells in these groups were caused by an enhanced ability of these cells to destroy the parasite. Sporozoites in untreated normal macrophages did not appear to be harmed by intracellular environment. After 30 minutes incubation, significantly fewer untreated immune macrophages contained sporozoites than untreated normal macrophages.

There was no statistically significant difference in the number of degranulated heterophils containing sporozoites

between normal and immune chickens. Fewer normal degranulated heterophils treated with immune serum contained sporozoites but there was no difference in the number of infected untreated immune heterophils compared with immune heterophils treated with immune serum. The lack of understanding of the degranulation phenomenon makes it difficult to interpret these findings.

On the basis of these findings, macrophages could play an important role in the resistance of chickens to reinfection with Eimeria tenella.

# CELLULAR ASPECTS OF THE RESISTANCE OF CHICKENS TO *EIMERIA TENELLA* INFECTIONS

By

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# INTRODUCTION

Eimeria tenella (Railliet and Lucet, 1891) is an intracellular protozoan parasite of chickens which invades the epithelial cells of the deep glands of the ceca. The Committee on Taxonomy and Taxonomic Problems of the Society of Protozoologists (1964) placed this organism in the class Telosporea under the subphylum Sporozoa. The presence of intracellular sporozoites is characteristic of the subclass Coccidia whose genera are separated on the basis of the appearance of the mature sporulated oocysts. Eimeria species are identified by an oocyst containing four sporocysts, each of which contains two sporozoites.

Levine (1961) described eight species of Eimeria which commonly infect the mucosa of the digestive tract of chickens. The cecal inhabitant, Eimeria tenella, is the most pathogenic of the eight. Unsporulated oocysts of E. tenella are passed in the feces of infected chickens. In the presence of oxygen, the oocysts sporulate and other chickens become infected by ingesting these sporulated oocysts. The sporulated oocyst contains four sporocysts each of which can release two sporozoites in the intestine. The sporozoites eventually invade the epithelium of the cecal glands of the host, where they round up and undergo schizogony, producing

merozoites which infect other cells and initiate a second generation of schizonts and merozoites. Although a few of these second generation merozoites may give rise to still a third generation of schizonts, the majority enter cells to become macrogametes or microgametes. Fertilization of macrogametes by microgametes results in the formation of an unsporulated oocyst which passes from the bird, sporulates, and is ready to initiate another cycle. The cycle is self limiting, lasting about 15 days from the time of ingestion to the appearance of the last oocysts.

Recovery from cecal coccidiosis confers a strong species specific immunity on the chicken. The mechanism of this immunity has been widely studied but it is still not understood. Antibodies have only recently been demonstrated in the serum of immune chickens, but all attempts to passively immunize chickens with immune sera have failed when the challenge has been by the normal route of infection. The demonstration by Van Doornick and Becker (1959), using Eimeria necatrix (Johnson, 1930), that macrophages engulf and transport the invading sporozoites through the lamina propria to the cells in which the parasite continues its cycle led Burns and Challey (1959) to suggest that an investigation of the role that the host's phagocytes play in effecting immunity could be profitably examined. Challey and Burns (1959) then showed that the macrophage acted as a

transporting agent in Eimeria tenella infections and participated in the life cycle at the approximate time that the host's immune response was activated.

It is the purpose of this study to investigate the role of the macrophage in the immune response of a chicken to infection with Eimeria tenella.

## REVIEW OF LITERATURE

Smith (1910) was probably the first to postulate a protective mechanism on the part of the host against an invading coccidian parasite. Working with intestinal coccidia of rabbits, he interpreted the observed infolding of epithelial cells to be a defense mechanism of the host which would isolate the gametes of the parasite and prevent fertilization. Hall and Wigdor (1918) infected a dog with Isospora bigemina (Stiles, 1891). Fourteen days after the feces of the dog were free of demonstrable oocysts, the dog was challenged with an oral inoculation of oocysts of the same strain. No oocysts were demonstrated in subsequent fecal examinations. These workers were unable to reinfect the dog with coccidia from its own feces, with the same oocyst suspension used in the initial infecting dose, or with coccidia of a totally different strain. The first clear proof of immunization to coccidia was demonstrated by Andrews (1926) who challenged a cat with a mixture of 100,000 oocysts of Isospora felis (Wenyon, 1923) and I. rivolta (Grassi, 1879) after an earlier sensitizing dose. Fecal examinations were negative for oocysts for 20 days and up to 78 days on subsequent challenges.

Johnson (1927) showed that chickens had a high degree

of resistance to coccidia fifteen days after a previous inoculation and Johnson (1928) also stated that there was no cross immunity between the various coccidia of the fowl.

The nature of the immunity to coccidiosis which develops in animals having recovered from the disease has not been clarified. In attempting to determine which stage of the life cycle is affected by the immune response, Morehouse (1938) immunized rats with Eimeria nieschulzi (Dieben, 1924). After challenging the immunized hosts, he was unable to find any developing stages in epithelial cells except in two cases out of a total of twelve, and sporozoites were found free in the intestinal lumen. Hammond, Heckman, Miner, Senger, and Fitzgerald (1959) challenged calves 30 to 35 days after a single immunizing dose of Eimeria bovis (Zublin, 1908). Intestinal scrapings and cross sections of the intestine were examined for schizonts and no consistent differences in concentration, total number, or size of schizonts between the immunized experimentals and non-immunized controls were found. They postulated that the immune response was directed against the merozoites or sexual stages of the life cycle. Hammond, Anderson, and Miner (1964) showed that no infection developed when merozoites of Eimeria bovis were inoculated into the ceca of immunized calves. They suggested a mechanism which did not destroy the parasite but one which inhibited its entry into a suitable

host cell.

The time of the parasitic life cycle that the immune mechanism which provides resistance to E. tenella is induced in chickens has been studied. The ellucidation of the coccidiostatic mechanism of sulfamethazine by several groups (Horton-Smith and Taylor, 1945, Farr and Wehr, 1947, Wehr and Farr, 1947) did much to clarify this problem. These studies showed that the second generation schizonts and merozoites were destroyed by treatment with sulfamethazine. Horton-Smith and Taylor (1945) showed that although sulfamethazine treatment stopped merogony of second generation schizonts, the chickens were fully resistant to challenge infections. Horton-Smith, Long, and Pierce (1963) introduced second generation merozoites rectally, producing an infection consisting almost entirely of processes of gametogenesis. Oocysts were produced but these chickens on a challenge oral infection with oocysts were not protected, the survivors producing oocysts in large numbers. In the same study, chickens infected rectally with sporozoites became populated with second generation schizonts, gametocytes, and oocysts which were released. These chickens on oral challenge with oocysts were completely protected. Both of these studies indicate that the sexual stages of the life cycle are not involved in the induction of the immune response to cecal coccidiosis in chickens.

Concerning the stage of the life cycle that the immune mechanism in a resistant bird is effective, Kendall and McCullough (1952) challenged birds at various times after a single immunizing dose of Eimeria tenella. Based on mortality, resistance was shown to be developing as early as 72 hours after the immunizing dose and when challenged at 96 hours after immunization, 22 percent of the immunized birds died as compared to 92 percent in the non-immunized control group. After experimentally immunizing chickens with three graded weekly doses of oocysts, Pierce, Long, and Horton-Smith (1962) detected no first generation schizogony occurring following a challenge infection. The fate of sporozoites in this study was in doubt since they were not detected either in immune or normal birds. Tyzzer, Theiler, and Jones (1932), working with E. necatrix and E. praecox, stated that the sporozoites in immune chickens were destroyed after invading the epithelium of the cecal glands. Horton-Smith, Long, and Pierce (1963) harvested sporozoites of E. tenella from an immune bird and used them to rectally infect a normal bird. These sporozoites initiated a typical infection resulting in a large output of oocysts. Sporozoites harvested from a normal bird and used to infect an immune bird resulted in the infection of the gland epithelia but the parasite was unable to undergo further development. Similar results were obtained when second generation mero-

zoites were used to infect immune chickens. Merozoites penetrated the gland epithelium of an immune chicken but were unable to develop further. These studies show that the protective response in a resistant chicken is active against the parasite after it has left the lumen of the intestine but before the development of first generation schizonts.

Serological studies on coccidiosis resistant animals were carried out by Bachman (1930a). He prepared antigens from the rabbit coccidia, Eimeria stiedae and E. perforans, and reacted them with sera from rabbits which had recovered from infections with the respective parasites. Although the results were not conclusive, complement fixing antibodies, but not precipitins, were demonstrated. A precipitin titer could be obtained if the serum was from artificially immunized rabbits.

Unsatisfactory results were obtained for many years when antibodies against chicken coccidia were sought. Tyzzer, Theiler, and Jones (1932) prepared an antigen from Eimeria necatrix. No antibodies were detected in sera from chickens immunized with the antigen nor could they be demonstrated in sera from chickens recovered from cecal coccidiosis. When E. tenella was used as a source of antigen, slight reactions were obtained, but the authors explained this as a response to impurities in the antigen preparation. McDermott and Stauber (1954) were the first to demonstrate agglutinins to

merozoites in the sera of birds resistant to Eimeria tenella. Itagaki and Tsubokura (1955) are also given credit for the demonstration of agglutination of merozoites but the serum used in the studies was from rabbits immunized against merozoites.

Rose and Long (1962) demonstrated precipitins in the sera of fowls which had been immunized with three graded weekly doses of oocysts of either Eimeria tenella, E. necatrix, E. acervulina, or E. maxima. Using the double diffusion in agar technique of Ouchterlony, all birds were shown to possess precipitins when their sera reacted with antigen obtained by repeated freezing and thawing of second generation merozoites. An 8% concentration of sodium chloride was used in the agar. Antibodies could still be demonstrated after a single challenge infection but not after a second challenge. The authors suggested that more sensitive methods of detection may be needed or that the parasite was unable to establish itself in the immune bird and insufficient antigen was produced to initiate a secondary antibody response. Pierce, Long, and Horton-Smith (1962) also demonstrated precipitins against E. tenella in the serum of immune fowl and, using electrophoresis, showed the antibody activity to be confined to the globulin fraction of lowest mobility.

Lysins were demonstrated by Long, Rose, and Pierce (1963). Both merozoites and sporozoites of Eimeria tenella

were lysed after one hour incubation at 37° C in homologous immune serum. No lytic activity of immune fowl serum was demonstrated after inactivation but the addition of fresh normal chicken serum to the inactivated immune serum restored its activity. Precipitins could not always be demonstrated in lytic serum. Horton-Smith, Long, Pierce, and Rose (1963) obtained similar results but noted that although no lysis occurred in heated immune serum, the merozoites appeared distorted after incubation. Burns and Challey (1965) showed that serum from birds became positive for lysins eight days following a moderate oral infection and suggested that first generation merozoites acted as the inducers of serum lysins. Unlike merozoites, sporozoites were lysed in normal serum.

Immune serum has been shown to inhibit the ability of sporozoites and merozoites to initiate infections. Merozoites incubated in immune serum produced infections greatly reduced in severity as compared to merozoites incubated in normal serum (Long, Rose, Pierce, 1963). Similar results were obtained when inactivated immune serum was employed. Sporozoites incubated in immune serum were also shown to produce less severe infections although sporocysts were not affected by immune serum. Herlich (1965) showed that sporozoites and merozoites of E. tenella and E. acervulina were immobilized in their respective immune sera and these immobilized stages failed to produce infections or produced

infections of a low magnitude.

Although antibodies which destroy stages of the coccidia or impair their ability to initiate severe infections have been demonstrated in serum from animals resistant to coccidiosis, attempts to passively transfer this immunity have failed. Tyzzer (1929) failed to protect normal chickens with immune serum administered subcutaneously, intraperitoneally, or per rectum. Neither were birds protected against challenge infections when fed blood from resistant donors. Bachmann (1930b) likewise failed to protect susceptible rabbits by the intravenous administration of immune serum and Becker (1935) similarly was unsuccessful in passively protecting newly infected rats by anti-Eimeria miyairii serum. Fitzgerald (1964) administered immune serum, concentrated by a freezing technique, to calves the same day of infecting with an oral inoculation of oocysts of Eimeria bovis. The serum injections were repeated on the seventh and fourteenth or only the fourteenth day following infection. Another experimental group received intraperitoneal injections of serum globulins every three days for two weeks. One week later, this group was challenged by the oral inoculation of sporulated oocysts. Based on oocyst output and the appearance of clinical symptoms, none of these calves showed any detectable differences with untreated controls.

Based on the number of oocysts in feces and macroscopic

or microscopic examination of the ceca, Pierce, Long, and Horton-Smith (1963) failed to transfer immunity to cecal coccidiosis. In these studies, whole serum was administered intravenously and intraperitoneally and challenge was by the oral inoculation of oocysts or the rectal inoculation of merozoites. Gamma globulin in quantities greatly exceeding the amount needed to protect chickens against Newcastle disease also failed to protect against challenge infections.

Long and Rose (1965) were, however, able to protect susceptible chickens if these birds were challenged with intravenously induced Eimeria tenella infections. The globulin fraction of serum from E. tenella resistant birds was given intravenously or intraperitoneally and these experimental birds were challenged with a suspension of sporozoites administered intravenously 8 hours before to 19 hours after the serum injections. Based on the oocyst output over several days or the number of oocysts in the ceca on the seventh day, those receiving immune globulin had much lighter infections. The protection was greater if the time between globulin administration and intravenous challenge was short. In this same study, birds actively immunized by an oral infection of oocysts or an intravenous injection of antigen derived from second generation schizonts, were protected when challenged with an intravenous injection of viable sporozoites.

The hypothesis that a coccidiosis resistant bird is protected by antibodies localized in the area of parasite development has been studied. Becker (1935) using glycerine and freezing methods to extract any antibody from the mucosa of rats resistant to Eimeria miyairii failed to release any protective principle. Herlich (1965) incubated sporozoites and merozoites in an extract of the cecal mucosa of hyperimmune birds. Although hyperimmune serum immobilized and inhibited these stages from initiating severe infections, cecal extracts appeared to have no demonstrable effects on these stages. Horton-Smith, Long, Pierce, and Rose (1963) report inconclusive tests which indicate that second generation merozoites incubated with a tissue extract obtained from the ceca of immunized birds give rise, on infection, to smaller numbers of oocysts than the controls. These extracts failed to show any precipitin bands in agar-gel techniques.

An understanding of the mechanism of the immunity to coccidiosis in birds became more difficult with the results of two similar studies which took advantage of the paired nature of the ceca in chickens. Burns and Challey (1959) attempted to eliminate any factors which might be involved in a localized immunity. This group isolated one of the cecal pouches by tying it off at its junction with the intestine. This isolated cecum was infected with a suspension of sporozoites of Eimeria tenella via a fistula, thus leaving

the non-ligated cecum unexposed to parasitic infection. After the first infection had subsided, these birds were challenged by an oral infection of oocysts and the non-ligated cecum was found to be just as resistant to challenge as the ligated cecum exposed to the primary infection. Horton-Smith, Beattie, and Long (1961) report similar results in a similar study, the difference being that the Horton-Smith group introduced the initial infection into the unligated cecum. The results indicated that a general systemic mechanism, presumably mediated by humoral antibodies, is involved in the mechanism of immunity to coccidiosis. In the historical development of the understanding of resistance to coccidiosis in chickens, these two studies provided the stimulus for the research on humoral antibody reported earlier in this review. However, they are also important starting points for a consideration of the cellular aspects of coccidian infections and immunity. Horton-Smith, Beattie, and Long (1961) indicate that although this transference of immunity might be mediated by humoral antibody, the transference could be effected by lymphoid cells of the host or a combination of both lymphoid cells and humoral antibody. Burns and Challey (1959) also state that judging from the large numbers of presumably phagocytic cells in the cecal mucosa that the role of these cells should be investigated with regards to a mechanism of resistance and the transfer

of immunity from one cecum to the other. Pierce, Long, and Horton-Smith (1962) state that the most prominent feature in the infection of normal birds with Eimeria tenella is the infiltration of heterophil leucocytes into the submucosa.

Hieronymus Fabricius, in 1621, described a diverticulum on the dorsal surface of the cloaca of chickens (Warren and Szenberg, 1964). It has been called the bursa of Fabricius and it resembles the thymus in that it reaches its greatest size in young animals and begins to regress at sexual maturity. Fabricius originally postulated that the organ served as a seminal recepticle. Glick, Chang and Jaap (1956) accidently discovered an immunological function for the bursa. Attempting to prepare a chicken antiserum to the O antigen of Salmonella, a group of birds which were left over from an experiment designed to ellucidate the function of the bursa were used for immunization. Only three of these bursectomized birds survived and none of the survivors produced antibodies to the Salmonella antigen. A group of normal birds survived the immunization procedures and built up normal antibody titers. In an experiment designed purposefully to investigate the immunological function of the bursa, 67 of 75 bursectomized birds while 10 of 73 intact birds failed to produce antibody to a specific antigenic stimulus.

Thorough studies on bursectomized or thymectomized chickens by Cooper, Peterson, South, and Good (1966) point

out the apparent dissociation of immune responsiveness in the chicken. In addition to the surgical removal of these organs, this group irradiated the experimental animals to eliminate any peripheral lymphoid components which may interfere with the interpretation of results. They found that only thymectomized chickens accepted skin homografts, were unable to exhibit delayed hypersensitivity, and showed less capacity to elicit a graft versus host response. In addition, there was a lowered blood lymphocyte level in thymectomized, but not bursectomized, chickens. Bursectomized chickens failed to produce serum antibodies to specific antigens but were competent in homograft reactions, delayed hypersensitivity, and graft versus host phenomena. The ability of the reticulo-endothelial system to clear colloidal gold was unchanged in birds following either bursectomy or thymectomy. In short, the thymus of chickens is responsible for the development of cell populations implicated in the phenomena associated with cell based immunity. The bursa of Fabricius is responsible for the development of cells of the plasmacyte series which are producers of antibody involved in humoral immunity.

That the bursa has a role in the mechanism of the immunity to coccidiosis was first postulated by Challey (1962). He found no difference between bursectomized and intact birds concerning adrenal gland size, adrenal ascorbic acid concen-

tration, or corticosterone concentrations following infections with Eimeria tenella. He did, however, report a markedly greater mortality in the bursectomized groups due to coccidiosis. He postulated that this might be involved with an impairment of the host's immune response and felt the results indicated that the bursa played a part in the host's resistance to coccidiosis. Long and Pierce (1963) were the first to utilize the technique of bursectomy to study the role of cellular factors in immunity to cecal coccidiosis in chickens. To arrest the development of the bursa, fertile eggs were injected with testosterone propionate on the 9th or 12th day of infection. Another group of control eggs was injected with the solvent used in preparing the testosterone solution. The eggs were hatched and some of each of the two groups were immunized with three graded doses of Eimeria tenella oocysts. The intact, immunized birds developed normal titers of lysins following immunizations. Bursectomized birds failed to develop antibodies following immunization, showed only an occasional pyroninophilic cell, and, as shown by electrophoresis and immunoelectrophoresis, lacked a slow moving globulin component of the serum. The lymphocyte picture in all birds was normal. All bursectomized, immunized birds were as resistant to a challenge infection as were intact, immunized birds even though their humoral antibody synthesizing powers had been destroyed. This same suspension

of oocysts produced severe infections in non-immunized groups.

The same results were obtained by Pierce and Long (1965) --bursectomized chickens were successfully immunized. This group also attempted to surgically remove the thymus of some birds. Complete thymectomy was rarely achieved. Birds which had undergone this thymectomy procedure produced antibody following immunization. There was, however, a significant reduction in the number of small lymphocytes in the blood. These chickens were successfully immunized against E. tenella but daily oocyst determination showed they were less resistant during immunization than normal birds. All birds were fully immune after immunization procedures.

In light of the recent results of bursectomy experiments, earlier studies involving the role of host macrophages and other phagocytic cells are of interest. Tyzzer (1929) occasionally found second generation schizonts in macrophages but considered this development to be anomalous. Gresham and Cruickshank (1959) found that the lamina propria of birds infected with Eimeria tenella was often entirely replaced by macrophages, many of which contained schizonts. It was unusual to find any signs of damage in these infected cells which were synthesizing protein which was apparently incorporated into the newly formed merozoites. Van Doorninck and Becker (1957) in studies with Eimeria necatrix, demonstrated that sporozoites passed through the intestinal mucosa to the

underlying lamina propria where many were engulfed by macrophages. The macrophages containing sporozoites which were apparently unharmed within the phagocyte then invaded epithelial cells where the sporozoites were released and developed into schizonts. Pattillo (1959) described the penetration of sporozoites through the epithelium via passageways which he termed "penetration tubes". The parasites entered the lamina propria where they invaded, or were engulfed by cells, which were probably macrophages. The sporozoites then invaded and initiated infections in the epithelium. Challey and Burns (1959) described the transportation of E. tenella sporozoites by macrophages through the lamina propria to the cells of the cecal glands where they initiated infections.

The involvement of some transport mechanism, possibly macrophages, is implied in the several attempts at parenteral infection of coccidiosis involving rats (Landers, 1960), and chickens (Davies and Joyner, 1962, Sharma, 1964, Long and Rose, 1965). Landers (1960) injected oocysts of Eimeria nieschulzi into rats intraperitoneally and intramuscularly. Oocysts were passed in the feces of these rats $7\frac{1}{2}$ days later. Intravenous, intramuscular, intraperitoneal and subcutaneous injections of oocysts or sporozoites of Eimeria tenella gave rise to schizogony in the ceca on the fifth day of infection and the passage of viable oocysts in the feces seven days after infection (Davies and Joyner, 1962, Sharma, 1964, Long

and Rose, 1965). These infections were usually less severe than oral infections produced by the same number of parasites.

That phagocytes assist as transport vehicles in invading hosts is not a phenomenon associated exclusively with coccidial infections. Goodpasture and Anderson (1937) infected the chorioallantoic membrane of embryonating chickens with various pathogenic bacteria. They found that most of these bacteria grew extracellularly in the presence of necrosis but they did not seem to be able to invade the living tissues of the embryo except by means of viable cells as vehicles. In an attempt to determine the fate of Plasmodium gallinarium, Huff and Coulston (1944) infected chickens with the salivary glands of infected mosquitoes. The first intracellular forms of the parasite were found within the first hour of the infection. The cells involved were macrophages and lymphoid cells in transition to macrophages. The parasite grew in these macrophages producing merozoites which invaded other macrophages. The authors stated that, at least for this particular species of Plasmodium, parasitism of blood cells must be preceeded by development in macrophages.

The macrophage has recently been implicated in more general immunological activities. Uhr and Weissman (1965), after injecting phage $\phi$X174 into rabbits, homogenized and fractionated the spleens and other lymphoid organs of the experimental rabbits. They found large quantities of the

bacteriophage associated with the lysosomes, as described by De Duve (1963), of cells of the reticulo-endothelial system. These lysosomal fractions were antigenic and antibody against ØX174 could be detected in animals immunized with the fraction. The authors concluded that an antigen must be degraded by the lysosomes of reticulo-endothelial cells before antibody formation is stimulated. The breakdown of an antigen, the M protein of streptococci, in macrophages was followed by Gill and Cole (1965) using fluorescent labeling techniques. They suggested that the recognition of self and non-self may be at the level of the host phagocyte rather than at the level of antibody synthesizing cells.

The production of antibodies in vitro has indicated the macrophage as an important participant in immune phenomena. Fishman (1961) was able to show the synthesis of antibodies in vitro only when the lymph node cell cultures used were incubated with a homogenate of macrophages which had earlier engulfed the antigen, $T_2$ bacteriophage. No antibodies were detected against the bacteriophage if the cell cultures were incubated with the phage or macrophages separately. Friedman, Stavitsky, and Solomon (1965) confirmed the Fishman work and, in addition, demonstrated the production of antibodies to the internal proteins of the phage. This demonstrated that the antigen had been modified within the macrophage.

That cells alone can mediate an immune mechanism in

vitro without the presence of immune serum has been demonstrated in several studies. Luria (1942) showed that active tuberculosis conferred on mononuclear phagocytes increased bacteriostatic properties for the tubercle bacillus. Bacteria invaded phagocytes from susceptible hosts, multiplied within those cells and eventually destroyed them. On the other hand, phagocytes derived from immune animals inhibited the growth of the intracellular bacilli and were not destroyed by them. This property of the cells was independent of immune body fluids to which the cells were exposed during the course of the infection. Saito and Mitsuhashi (1965) and Sato and Mitsuhashi (1965) showed that monocytes from hyperimmunized mice inhibited the intracellular growth of virulent Salmonella enteritidis and were not destroyed by the bacteria. The effect was independent of immune serum in the media in which the cells were growing. This cellular immunity could be transferred to cells derived from normal animals by incubating the normal cells with the media which immune cells were grown. Transfer was inhibited by ribonuclease. The ribosomal fraction from immune cells was also capable of transferring immunity when incubated with normal cells.

That immune serum enhances the ability of a host's phagocytic cells has also been reported. Bonventre and Oxman (1965) used a liver perfusion system to investigate the

ability of immune or normal livers to clear the perfusate of non-immunogenic Staphylococcus aureus or immunogenic Salmonella enteritidis. The clearance of Staph. aureus from the perfusate and its survival in reticulo-endothelial cells of the liver were unaffected whether the liver was from an immunized rat or a normal rat. The nature of the serum involved, immune or normal, also had no effect. Salmonella enteriditis was sensitive to both the cellular and humoral immune factors which could be controlled in this interesting technique. There was some degree of intracellular inactivation of this organism when an immune liver was perfused with normal serum. However, immune serum perfused through a normal liver enhanced phagocytosis and inhibited intracellular growth as well as affording a limited degree of bacterial destruction.

Work on cellular factors involved in immunity to protozoan infections is limited and usually involves the coating of the organisms, or infected host cells, with antibody which enables the hosts phagocytes to engulf it. Zuckerman (1945) suspended red blood cells infected with either Plasmodium gallinaceum or Plasmodium lophurae in normal or hyperimmune serum. These cells were then incubated for a standard length of time with cultured macrophages. Those cultures exposed to infected blood cells in normal serum showed only sluggish phagocytic activity. Those

macrophages exposed to immune serum treated red blood cells were surrounded by adherant erythrocytes and numerous red cells had been ingested by the macrophages which were actively digesting the infected cells. No reports concerning the activity of macrophages derived from immune animals were available from this study. Suter and Ramseier (1964) report on a 1954 study by Vischer and Suter on Toxoplasma gondii, an obligate intracellular protozoan parasite. Macrophages from an animal which has survived an infection with an attenuated strain of Toxoplasma support only limited growth. This latter effect is greatly enhanced by the presence of immune serum.

Only one attempt at transferring immunity to Eimeria tenella by the use of immune cells has been reported. Horton-Smith and Long (1963) obtained white blood cells from the peripheral circulation of immune and non-immune birds. These cells were administered intravenously to two groups of chickens four days before and at the time of a per rectum infection with second generation merozoites. The production of oocysts from those birds receiving immune cells was not significantly different from the oocyst production of birds receiving normal white blood cells.

## METHODS AND MATERIALS

### Obtaining and Maintenance of Chickens:

On the day of hatching, chickens were obtained from Gulliver's Hatchery, Eaton Rapids, Michigan. In some cases, day old chickens were also obtained from the Department of Poultry Science, Michigan State University. Eighteen day old embryonating eggs were obtained from the diagnostic laboratory of the College of Veterinary Medicine at Michigan State University and hatching was completed at 37° C. Chickens were housed in brooders with wire mesh floors in a walk-in incubator kept at 37° C and were fed a ration containing no antibiotics or coccidiostats. Chickens infected with *Eimeria tenella* or chickens which had been immunized against *E. tenella* were kept in a separate room. At no time were coccidian oocysts or infected chickens brought into the room housing normal chickens and inoculation of chickens with oocysts was performed in the room in which infected chickens were housed. None of the chickens housed in the "normal" room showed clinical symptoms of cecal coccidiosis, and during the course of the studies reported here examination of feces passed by these chickens never revealed the presence of oocysts.

Obtaining and Maintenance of Eimeria tenella:

A suspension of sporulated oocysts of Eimeria tenella (Strain 8) was obtained from the Eli Lilly Laboratories, Greenfield, Indiana. This strain was maintained in the parasitology laboratory at Michigan State University by periodically infecting chickens, which had fasted for 24 hours, with 30,000 to 40,000 sporulated oocysts. Seven days later, these chickens were killed and their ceca removed. The ceca and their contents were disrupted in a Waring blender for 30 seconds and then incubated for 3 hours in .1N NaOH (Long and Rose, 1965). At the end of that time, the mixture was diluted 10 times with water and strained through a United States Bureau of Standards #80 seive, centrifuged, and the sediment added to a solution of 2.5% potassium dichromate. This suspension was poured into the bell of a large funnel supported on a ring stand. An aquarium air stone was fitted in the stem of the funnel and connected to an air jet. The suspension was thus aerated for 48 hours. The resulting dichromate suspension of sporulated oocysts was centrifuged and the sediment was cleaned by repeated washing and centrifugation. A water suspension of these oocysts was diluted 1:1 with hypochlorite which removed tissue debris contaminating the suspension. The cleaned oocysts were washed free of hypochlorite and the number of oocysts in the resulting tap water suspension was determined

using a hemocytometer. The oocysts were stored at 4° C.

Collection of Sporozoites:

The method of Farr and Doran (1962) was used for obtaining sporozoites. A saline solution of 5% chicken bile and .25% trypsin 1-300 (Nutritional Biochemicals) was prepared at a pH of 7.3 to 7.6. Bile was asceptically collected from adult chickens, diluted to 5% in sterile saline and frozen at -20° C. The required amount of trypsin was added to the bile solution to make it 0.5% with regard to trypsin when the excysting fluid was needed. This solution was sterilized by filtration through a Millipore filter with 0.2 micron pore size. A suspension of clean oocysts of Eimeria tenella, sterilized in 3% peracetic acid for 30 minutes and washed with sterile distilled water, was shaken with sterile beads in a sterile 50 ml plastic centrifuge tube for 30 minutes. The released sporocysts were concentrated by centrifugation and incubated in the bile-trypsin excysting fluid for 3 hours at 41° C or 5 hours at 37° C depending on when the sporozoites were needed. The released sporozoites were washed free of the excysting fluid with two changes of Hanks' balanced salt solution (Microbiological Associates) and used within four hours of collection. The trypsin-bile excysting fluid, if stored after use at -20° C, was active up to a week. At the end of that time, a fresh solution was prepared.

Immunization of Chickens:

Chickens were artificially immunized following the method of Pierce, Long, and Horton-Smith (1962). Chickens, which had fasted for 24 hours, were infected per os with 500, 5,000, and 50,000 oocysts respectively for three consecutive weeks. If not used immediately, these birds were given challenge infections of 100,000 oocysts at ten week intervals. The immunized birds had no clinical signs of coccidiosis after these challenge infections.

Collection of Immune Serum:

Blood was obtained asceptically by heart puncture of adult chickens which had been immunized following the protocol outlined above. The blood was allowed to coagulate for one hour at 37° C. After this time, the clot was freed from the sides of the collecting vial and stored over night at 4° C. Following this period, the coagulated blood was centrifuged and the serum collected and stored in sterile vials at -20° C.

Normal Serum:

Normal chicken serum was obtained commercially from Microbiological Associates.

Peritoneal Exudates:

Numerous methods to stimulate peritoneal exudates in various animals have been successfully employed by various researchers. Glycogen (Wu and Marcus, 1963), beef heart infusion broth with proteose peptone (Fishman, 1961), starch suspensions (Bennett, Old, and Boyce, 1964), gum arabic (Robertson and Van Sant, 1964), and Hanks' balanced salt solution (Miller, 1966) have been reported. In addition to these solutions, gastric mucin, mineral oil, an antigen of Salmonella pulorum (Vineland), and a suspension of rice powder were respectively injected intraperitoneally into groups of chickens and the resulting exudates were harvested at 24 and 48 hours after the injection. Some, such as gastric mucin, did not yield large quantities of cells. The starch suspension stimulated large exudates but the cells had engulfed large quantities of starch particles which could affect their subsequent phagocytic activities. All chickens receiving beef heart infusion broth with proteose peptone were dead within 24 hours. It was decided that Hanks' balanced salt solution (BSS) gave rise to the largest number of phagocytic cells and since the components of Hanks' BSS may be metabolized in the cells, this material would less probably affect their phagocytic and post phagocytic activities.

Peritoneal exudates were stimulated in 2-4 week old

chickens by intraperitoneal injections of 2-5 cc of Hanks' BSS (Microbiological Associates) 48, 24 and 2-6 hours before cells were to be harvested. A sterile syringe, fitted with a #22 needle, was used. Before injecting the salt solution, the plunger of the syringe was drawn back to insure the needle had not been inserted into an air sac. When the cells were to be harvested, the birds were killed by cervical dislocation and the skin was removed from the posterior half of the bird to avoid the presence of feathers in the cell preparation. A 1-2 centimeter incision was made in the ventral body wall and a variable quantity of Hanks' BSS was poured into the exposed coelom. The body wall and intestines were gently washed and agitated with the aid of a sterile cotton swab and the washings were poured through the incision directly into a sterile petri plate. The suspension of cells was then pipetted into a sterile 15 ml centrifuge tube and centrifuged at 1000 rpm for 5 minutes. The pellet of cells was resuspended in 1-2 ml of Hanks' BSS and approximately 0.5 ml of the suspension was pipetted onto coverslips which were ringed with vasalene. The cells were allowed to settle out from the suspension and attach to the coverslip for 15 minutes at 41° C. The attached cells were washed free of unattached cells, erythrocytes, and other debris by gently pipetting a small quantity of Hanks' BSS over the surface of the preparation. The coverslip was inverted on a clean glass

slide and observed under an oil immersion objective using conventional light or phase microscopy.

Experimental Procedures:

Experiment 1--Twenty newly hatched white leghorn chickens were allotted into 2 groups of 10. One of these groups was immunized by graded weekly *per os* inoculations of sporulated oocysts of *Eimeria tenella*. The other group was maintained coccidia free in similar quarters. Each of the two groups was randomly divided into two groups of five. A group of five immunized and five normal chickens received intraperitoneal injections of Hanks' BSS to stimulate a peritoneal exudate and 48 hours after the first injection of BSS, one million sporozoites of *Eimeria tenella* were injected intraperitoneally. After 15 minutes, the exudate was harvested and the cells were examined microscopically for the presence of sporozoites. A total of 500 cells from each chicken was counted by randomly moving the preparation under the microscope but trying to assure that all regions of the preparation were examined. The experiment was repeated with the second series of five chickens in each group.

Experiment 2--Of the several cell types which could be identified in the exudates of the chickens in Experiment 1, only two were found to contain sporozoites-macrophages and degranulated heterophil leucocytes. In light of this finding,

Experiment 2 was carried out in an identical fashion to Experiment 1 but only the macrophages and degranulated heterophils in the exudate were counted.

Experiment 3--The effect of serum on exudate cells and the number of cells subsequently found to contain sporozoites was determined in Experiment 3. Two ml of immune or normal serum were injected into the coelom of normal and immune chickens during the process of stimulating the exudate. Serum was injected 12 hours and 36 hours after the first injection of BSS. This would bring the exudate cells in contact with the sera but the sera would be diluted or equilibrated by the host before sporozoites were introduced into the chicken. The experiment was carried out twice with groups of 5 chickens.

Experiment 4--The effect of a longer length of contact between sporozoites was examined in Experiment 4. The techniques were identical to those reported above except that the sporozoites were within the stimulated peritoneal cavity for 30 minutes and only 5 chickens were used in each group in the experiment.

Experiment 5--To determine the effect of the coelomic environment on the sporozoites, a 3 ml suspension of sporozoites was injected into the unstimulated peritoneal cavity of normal and immune chickens. After 15 minutes, the contents of the coelom were washed out following the procedure used to

harvest exudate cells and the number of sporozoites recovered was determined by hemacytometer counts of 3 samples of each of the washings.

Statistical Analysis of Data:

The t test (Guenther, 1964) was chosen to statistically evaluate the mean percent infected cells of the various experimental groups. It is a valid test to use on means obtained from sample populations of less than 30 and takes into account the differences observed in the variances of the various experimental groups. A 2.5% level of confidence was chosen in all cases and there is probably little question of the validity of the results of those groups which were statistically significant under the experimental conditions imposed on the system. Those results which were not significant under these experimental conditions might be statistically significant at the same level of confidence under other conditions such as increased sample sizes.

The F test (Guenther, 1964) was used to statistically evaluate differences in variances among the groups.

Photography:

The appearance of the exudate and the sporozoites within macrophages and degranulated heterophil leucocytes was recorded on Kodak Plus-X film using an Eastman Kodak 35 mm camera.

## RESULTS

The combined results of the two replicate sets of Experiment 1, with percentages based on the entire exudate population, are given in Table I. An unusually low yield of cells in birds number 16, 19, and 28 resulted in fewer than 500 cells being counted although the entire area of the preparation was examined.

The results of Experiment 1 were of interest from several aspects. First, the difference in the mean number of infected cells between the two groups of chickens was not significant at the 2.5 percent level of confidence. Second, the appearance of the sporozoites in cells derived from immune birds was generally different from their appearance in cells from a susceptible chicken (Figures 1, 2, 3, and 4). The majority of sporozoites in normal cells were easily distinguished and appeared to be little changed in morphology from those motile sporozoites which could occasionally be seen outside the cells. Third, of the several cell types which could be identified in the exudates of these chickens, only two were found to contain sporozoites. One of these was a large cell which contained an oval to bean shaped nucleus which stained a red-violet color with the May-Grunwald-Giemsa technique (Merchant, Kahn, and Murphy, 1964). The cytoplasm

Table I. Number of chicken peritoneal exudate cells found to contain sporozoites in normal and immune birds

| Normal Birds | | | | Immune Birds | | | |
|---|---|---|---|---|---|---|---|
| Bird number | Total Cells | Infected Cells | Percent of Infected Cells | Bird Number | Total Cells | Infected Cells | Percent of Infected Cells |
| 15 | 505 | 5 | .1 | 25 | 509 | 3 | .6 |
| 16 | 350 | 12 | 3.0 | 26 | 502 | 1 | .2 |
| 17 | 512 | 7 | 1.5 | 27 | 514 | 5 | 1.0 |
| 18 | 501 | 15 | 3.0 | 28 | 253 | 5 | 2.1 |
| 19 | 320 | 6 | 1.9 | 29 | 545 | 0 | 0 |
| 20 | 513 | 12 | 2.3 | 30 | 503 | 7 | 1.4 |
| 21 | 517 | 2 | .4 | 31 | 476 | 11 | 2.3 |
| 22 | 500 | 24 | 4.8 | 32 | 406 | 12 | 2.9 |
| 23 | 510 | 19 | 3.7 | 33 | 504 | 6 | 1.2 |
| 24 | 504 | 13 | 2.6 | 34 | 507 | 5 | 1.0 |
| Mean percent of infected cells | | | 2.3 | Mean percent of infected cells | | | 1.3 |
| | | Variance | 2.0286 | | | Variance | .8645 |

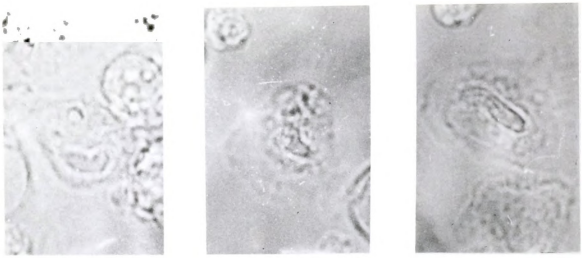

Figure 1. Macrophages derived from immune chickens containing sporozoites of Eimeria tenella.

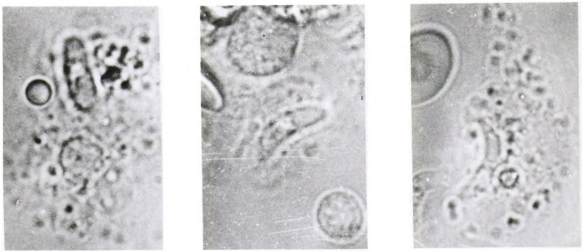

Figure 2. Macrophages derived from normal chickens containing sporozoites of Eimeria tenella.

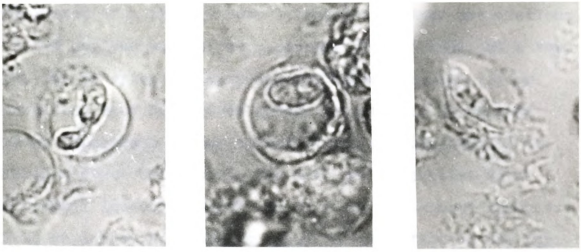

Figure 3. Degranulated heterophil leucocyte (ring cells) derived from immune chickens containing sporozoites of Eimeria tenella.

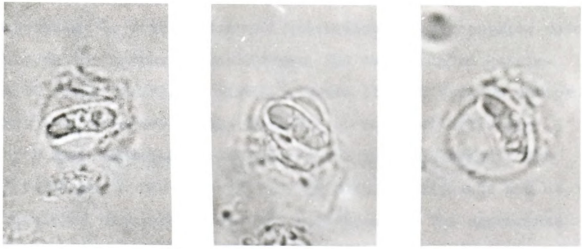

Figure 4. Degranulated heterophil leucocytes (ring cells) derived from normal chickens containing sporozoites of Eimeria tenella.

of these cells in stained preparations appeared highly vacuolated and stained a light blue color. With the aid of Atlas of Avian Hematology (Lucas and Jamroz, 1961), this cell was identified as a macrophage. The second cell had the appearance of a ring in both living and stained preparations. A lobed nucleus was at the periphery of this cell and a very thin rim of cytoplasm surrounded a large vacuole. After consulting with Dr. Alfred Lucas, director of the United States Department of Agriculture Avian Anatomy Project at Michigan State University, the cell was identified as a heterophil leucocyte which had lost its rod shaped granules. Although intact heterophils could be identified in the peritoneal exudates, they never were demonstrated to contain sporozoites until they had lost their granules and assumed the ring appearance.

The combined results of the two replicate sets of Experiment 2, with 15 minutes incubation and percentages based only on the number of macrophages and degranulated heterophils (ring cells), are given in Table II. It will be noticed that the variances in the percents of infected cells are quite high especially with regards to the ring cells. The differences in the mean percent infected macrophages and degranulated heterophils are not significant. The appearance of the sporozoites within the cells was similar to that found in Experiment 1.

Table II. Percentage of peritoneal exudate cells of immune and normal chickens containing sporozoites of Eimeria tenella - 15 minute incubation

| Bird Number | Total Macrophages | Infected Macrophages | Percent of Infected Macrophages | Total Ring | Infected Ring | Percent of Infected Ring |
|---|---|---|---|---|---|---|
| **Normal** | | | | | | |
| 35 | 281 | 22 | 7.8 | 240 | 27 | 11.2 |
| 37 | 420 | 13 | 3.1 | 82 | 7 | 8.5 |
| 38 | 458 | 16 | 3.5 | 47 | 7 | 14.9 |
| 39 | 365 | 2 | .5 | 135 | 2 | 1.5 |
| 40 | 316 | 28 | 8.9 | 186 | 21 | 11.3 |
| 70 | 376 | 45 | 12.0 | 128 | 10 | 7.8 |
| 71 | 451 | 27 | 6.0 | 52 | 6 | 11.5 |
| 72 | 459 | 16 | 3.5 | 51 | 2 | 4.0 |
| 73 | 373 | 18 | 4.8 | 133 | 4 | 3.0 |
| 74 | 406 | 24 | 5.9 | 95 | 17 | 17.9 |
| Mean percent of infected cells | | | 5.6 | | | 9.16 |
| Variance | | | 13.2956 | | | 27.676 |
| **Immune** | | | | | | |
| 41 | 404 | 4 | 1.0 | 6 | 0 | 0 |
| 42 | 458 | 9 | 2.0 | 48 | 10 | 20.8 |
| 43 | 490 | 20 | 4.1 | 10 | 6 | 66.7 |
| 44 | 386 | 30 | 7.8 | 111 | 10 | 9.0 |
| 45 | 512 | 5 | 1.0 | 5 | 1 | 20.0 |
| 46 | 493 | 7 | 1.4 | 7 | 2 | 28.6 |
| 47 | 495 | 52 | 10.7 | 5 | 2 | 40.0 |
| 48 | 487 | 41 | 8.4 | 14 | 2 | 14.3 |
| 103 | 325 | 1 | .3 | 181 | 0 | 0 |
| 104 | 296 | 1 | .3 | 222 | 2 | .9 |
| Mean percent of infected cells | | | 3.7 | | | 21.0 |
| Variance | | | 13.5277 | | | 495.91 |

The combined results of the two replicate sets of the two parts of experiment 3 are given in Table III, the effects of normal serum, and Table IV, the effects of immune serum. There were significantly fewer infected immune macrophages treated with immune serum than infected normal macrophages treated with immune serum. There was no significant difference between the number of infected immune or normal ring cells when treated with immune serum but significantly fewer normal ring cells than immune ring cells were observed to be infected when the cells had been treated with normal serum.

Table V gives the results obtained when the sporozpoites and exudate cells were in contact with each other for 30 minutes within the coelom. There were significantly fewer immune cells than normal cells found to contain sporozoites.

Table VI shows the percent recovery of sporozoites from the peritoneal cavity of chickens which had not received BSS to stimulate the development of an exudate. All sporozoites were actively motile and did not appear to be harmed by the coelomic environment or the manipulations involved in their recovery which were identical to the manipulations involved in harvesting exudate cells.

Table VII and Table VIII show the t value obtained when the mean percent of infected cells from any two experimental groups was calculated. The calculations were based on a 2.5 percent level of significance.

Table III. Percentage of normal serum treated peritoneal exudate cells from immune and normal chickens containing sporozoites of Eimeria tenella - 15 minute incubation

| Bird Number | Total Macrophages | Infected Macrophages | Percent of Infected Macrophages | Total Ring | Infected Ring | Percent of Infected Ring |
|---|---|---|---|---|---|---|
| **Normal** | | | | | | |
| 85 | 146 | 3 | 2.0 | 479 | 0 | 0 |
| 86 | 226 | 5 | 2.2 | 285 | 1 | .4 |
| 87 | 220 | 2 | .9 | 281 | 2 | .7 |
| 88 | 299 | 2 | .7 | 206 | 0 | 0 |
| 89 | 271 | 3 | 1.1 | 237 | 0 | 0 |
| 90 | 397 | 4 | 1.0 | 111 | 3 | 2.7 |
| 91 | 505 | 1 | .2 | 13 | 1 | 7.7 |
| 92 | 342 | 1 | .3 | 165 | 3 | 1.2 |
| 93 | 259 | 2 | .8 | 243 | 1 | .4 |
| 94 | 416 | 3 | .7 | 89 | 1 | 1.1 |
| Mean percent of infected cells | | | 1.0 | | | 1.4 |
| Variance | | | .4232 | | | 5.3325 |
| **Immune** | | | | | | |
| 50 | 350 | 6 | 1.7 | 154 | 6 | 3.9 |
| 51 | 443 | 14 | 3.2 | 63 | 1 | 1.6 |
| 52 | 492 | 9 | 1.8 | 25 | 0 | 0 |
| 53 | 358 | 4 | 1.1 | 156 | 0 | 0 |
| 54 | 477 | 4 | .8 | 35 | 2 | 5.7 |
| 55 | 390 | 6 | 1.5 | 69 | 4 | 5.8 |
| 56 | 294 | 6 | 2.0 | 222 | 3 | 1.4 |
| 57 | 386 | 4 | 1.0 | 120 | 5 | 4.2 |
| 58 | 266 | 4 | 1.5 | 241 | 1 | .4 |
| 59 | 465 | 6 | 1.3 | 48 | 5 | 10.4 |
| Mean percent of infected cells | | | 1.6 | | | 3.3 |
| Variance | | | 12.574 | | | .4588 |

Table IV. Percentage of immune serum treated peritoneal exudate cells from normal and immune chickens which contained sporozoites of Eimeria tenella - 15 minute incubation

| Bird Number | Total Macrophages | Infected Macrophages | Percent of Infected Macrophages | Total Ring | Infected Ring | Percent of Infected Ring |
|---|---|---|---|---|---|---|
| **Normal** | | | | | | |
| 75 | 483 | 31 | 6.4 | 23 | 1 | 4.3 |
| 76 | 438 | 20 | 4.1 | 81 | 2 | 2.5 |
| 77 | 483 | 16 | 3.3 | 17 | 1 | 5.9 |
| 78 | 449 | 8 | 1.8 | 59 | 3 | 5.1 |
| 79 | 375 | 7 | 1.9 | 136 | 2 | 1.5 |
| 80 | 363 | 13 | 3.6 | 139 | 7 | 5.0 |
| 81 | 375 | 6 | 1.6 | 126 | 1 | .8 |
| 82 | 451 | 9 | 2.0 | 55 | 2 | 3.6 |
| 83 | 353 | 16 | 4.5 | 148 | 18 | 12.2 |
| 84 | 343 | 3 | .9 | 163 | 1 | .6 |
| Mean percent of infected cells | | | 3.0 | | | 4.2 |
| | | Variance | 3.1048 | | | 11.4872 |
| **Immune** | | | | | | |
| 60 | 460 | 0 | 0 | 44 | 1 | 2.3 |
| 61 | 475 | 11 | 2.3 | 33 | 0 | 0 |
| 62 | 494 | 3 | .6 | 14 | 1 | 7.1 |
| 63 | 386 | 0 | 0 | 19 | 0 | 0 |
| 64 | 487 | 2 | .4 | 22 | 0 | 0 |
| 65 | 342 | 0 | 0 | 158 | 1 | .6 |
| 66 | 481 | 3 | .6 | 21 | 1 | 4.8 |
| 67 | 497 | 2 | .4 | 13 | 3 | 23.1 |
| 68 | 503 | 4 | .8 | 17 | 0 | 0 |
| 69 | 479 | 3 | .6 | 29 | 1 | 11.1 |
| Mean percent of infected cells | | | .6 | | | 4.9 |
| | | Variance | .4534 | | | 55.0944 |

Table V. Percentage of peritoneal exudate cells from immune and normal chickens containing sporozoites of *Eimeria tenella* - 30 minute incubation

| Bird Number | Total Macrophages | Infected Macrophages | Percent of Infected Macrophages | Total Ring | Infected Ring | Percent of Infected Ring |
|---|---|---|---|---|---|---|
| **Normal** | | | | | | |
| 101 | 303 | 9 | 3.0 | 199 | 10 | 5.0 |
| 102 | 335 | 5 | 1.5 | 167 | 9 | 5.4 |
| 108 | 384 | 3 | .8 | 123 | 5 | 4.1 |
| 109 | 436 | 5 | 1.1 | 73 | 2 | 2.7 |
| 111 | 440 | 5 | 1.1 | 68 | 12 | 17.7 |
| Mean percent of infected cells | | | 1.5 | | | 7.0 |
| Variance | | | .735 | | | 36.67 |
| **Immune** | | | | | | |
| 112 | 372 | 1 | .27 | 139 | 3 | 2.2 |
| 113 | 276 | 0 | 0 | 269 | 1 | .4 |
| 114 | 417 | 1 | .23 | 83 | 0 | 0 |
| 115 | 365 | 1 | .27 | 142 | 1 | .7 |
| 116 | 430 | 1 | .23 | 76 | 1 | 1.3 |
| Mean percent of infected cells | | | .20 | | | .9 |
| Variance | | | .013 | | | .715 |

Table VI. Recovery of sporozoites from unstimulated peritoneal cavity of normal and immune chickens

| Number | Normal or Immune | Sporozoites Injected | Sporozoites Recovered (mean of 3 counts) | Percent of Sporozoites Recovered |
|---|---|---|---|---|
| 1 | Normal | 2,250,000 | 650,000 | 28 |
| 2 | Normal | 2,250,000 | 882,000 | 36 |
| 3 | Normal | 1,500,000 | 500,000 | 34 |
| 4 | Immune | 290,000 | 243,000 | 84 |
| 5 | Immune | 290,000 | 120,000 | 41 |

Table VII. Student's t value
obtained by comparing mean percent infected macrophages
of any two experimental groups at 2.5% level of confidence

| | Immune Cells | Normal Cells Normal Serum | Normal Cells Immune Serum | Immune Cells Normal Serum | Immune Cells Immune Serum |
|---|---|---|---|---|---|
| Normal Cells | 1.0988 | 3.73* | 1.919 | 3.234* | 3.826* |
| Immune Cells | X | 2.1865* | .51497 | 1.7007 | 2.5195* |
| Normal Cells Normal Serum | X | X | 3.254* | 1.197 | 4.255* |
| Normal Cells Immune Serum | X | X | X | 2.257* | 3.881* |
| Immune Cells Normal Serum | X | X | X | X | 3.204* |

* Statistically significant at 2.5% level of confidence ($t_{2.5\%} = 2.101$).

Table VIII. Student's t values
obtained by comparing the mean percent infected ring cells
of any two experimental groups at 2.5% level of confidence

| | Immune Cells | Normal Cells Normal Serum | Normal Cells Immune Serum | Immune Cells Normal Serum | Immune Cells Immune Serum |
|---|---|---|---|---|---|
| Normal Cells | 1.556 | 4.047* | 2.402* | 2.752* | 1.405 |
| Immune Cells | X | 2.629* | 2.248* | 2.354* | 2.062 |
| Normal Cells Normal Serum | X | X | 1.988 | 3.369* | 1.347 |
| Normal Cells Immune Serum | X | X | X | 4.954* | .276 |
| Immune Cells Normal Serum | X | X | X | X | .559 |

* Statistically significant at 2.5% level of confidence ($t_{2.5\%} = 2.101$).

# DISCUSSION

Before discussing the results of these experiments and their implications it is important to comment on the techniques as a whole. Although the actual site of cell-parasite interaction in these studies was not the same site that such interactions would occur in a natural infection of Eimeria tenella, it is an in vivo technique and offers conditions closer to those of the natural site of infection than does an in vitro situation. This offers certain advantages, however, the lack of control which the experimenter has over the conditions is a definite disadvantage. The coelomic environment, such as its antibody content, could vary greatly from chicken to chicken and thus influence the results obtained.

Rowley (1962) has outlined several criticisms of studies of phagocytosis which are, for the most part, avoided in these experiments. Since the experimental technique used in these studies is an in vivo technique, the use of antibiotics which might affect the phagocytic activity of the cells or the fate of the sporozoites themselves is completely avoided in these studies. Secondly, although the harvested exudate cells were sedimented by centrifugation, and resuspended in Hanks' balanced salt solution (BSS), a treatment which could

affect cellular vitality, phagocytosis of the parasite had already occurred and this factor is thus no longer a problem in terms of determining the number of infected cells. Thirdly, the use of Hanks' BSS to stimulate a peritoneal exudate might affect the cell's phagocytic and post-phagocytic activities. This material is metabolized completely by the cells and is thus more suitable for these purposes than a starch solution or mineral oil which remains in the cells for some time. It should be remembered, however, that cells obtained by the use of any artificial stimulatory agent are no longer "normal" and may possess properties which might affect their activities. Finally, the rapid examination of the cells in this procedure reduces the liklihood of changes occurring in the cells as a result of their removal from the chicken and their culture for several days under artificial conditions.

The results of these experiments are presented in such a way that the cell populations under study are treated as if they were homogenous and the results obtained are based on individual cells. Various studies reported by Nossal (1962) concerning immunologically competent plasma cells indicate that these cells involved in antibody synthesis are morphologically indistinguishable but show a marked functional heterogeneity. Thus, although many plasmacytes may contact the same antigen, not all will respond in a similar manner.

There is no reason to suppose that a similar heterogeneity might not exist among cells involved in immunologic phenomena other than the actual production of immune globulins, such as phagocytosis or cellular immunity. The results of these experiments do not take into account the dynamic state of the cell populations involved. There may not only be differences in the immunologic or phagocytic activities between individual indistinguishable cells of the same population but there may also be differences within the same cell at various stages of its development.

Although a discussion of the two types of exudate cells which were shown to contain sporozoites will occur later in the paper, some general comments on the peritoneal exudate which was obtained should be made at this time. The percentages reported in the results are based only upon the particular cell types found to contain sporozoites and not on the total number of cells in the exudate. For the most part, the exudate obtained from chickens hyperimmunized by three weekly oral inoculations of Eimeria tenella gave much larger total yields of cells based on gross observation. Generally, the total increase was an expression of an increase in the numbers of intact heterophils which appeared in response to the balanced salt solution stimulation. A similar increase in total cell yield, again expressed primarily by increased heterophil infiltration, was observed when normal or immune

serum was injected during the period of exudate stimulation. This difference in total cell numbers would not be involved in altering the percentage of macrophages or degranulated heterophils containing sporozoites since these were the only two cell types counted. However, the fact that there may be more cells present in the exudates of some groups alters the environment in which phagocytosis is occurring and could in some way affect the phagocytic or post-phagocytic events.

The differences in the mean percent infected cells of both types in the various experimental groups may be explained in several ways. In the first place, these differences may be due entirely to chance. The use of the "t" test to analyze these means has indeed shown that some of the differences in the groups are a matter of chance and are therefore not the result of any experimental manipulations. Other differences in the means are significant under the conditions of the experiment as judged by the magnitude of the calculated "t" value.

In the second place, the sporozoites introduced into experimental groups with significantly lower mean percent infected cells may have been destroyed by extracellular factors in the coelom. These factors may have been released from the cells of the exudate in response to the inflammatory agent and, although not necessarily directed specifically against the sporozoites, may have had an adverse effect on

them. These destructive substances, such as antibody, may be present as normal components of the peritoneal fluid and be derived from the cells of the serous fluid in the chicken. In some of the experimental groups, immune or normal serum was introduced into the coelom at intervals such that the exudate cells would come in contact with it but that it would not be present in concentrations great enough to affect the sporozoites themselves which were introduced at least twelve and, in some cases, twenty-four hours after the last serum injection. It still may be that enough residual serum may have been present to destroy or adversely affect the sporozoites and serum lysins have been described in in vitro tests (Long, Rose, and Pierce, 1963, Burns and Challey, 1965). Furthermore, although some component of the peritoneal fluid may not visibly damage a sporozoite extracellularly it may render it more susceptible to destruction within an intracellular environment. In such a case, the sporozoites may not be found because of their rapid breakdown within the cell. Again, any residual serum in the coelom of those groups receiving serum during the development of the exudate may be involved here. Although these environmental factors of the peritoneal cavity could be involved in extracellular destruction of sporozoites, observations and experiments carried out tend to argue against this possibility. In the test reported, 30 to 35 percent of the sporozoites could be

accounted for and recovered from the unstimulated coelom of chickens. In one case, 85 percent of the sporozoites could be recovered from the peritoneal cavity of an immune bird after fifteen minutes. Even the lowest recovery rate of 28 percent is fairly good since there was not a deliberate attempt to recover them but simply to determine the recovery of sporozoites following a procedure similar to the harvesting of the exudate cells. These recovered sporozoites did not differ morphologically from sporozoites not subjected to the coelomic environment and were actively motile. Also, with some exceptions, the cell preparations studied from each individual bird contained a few actively motile, intact sporozoites moving about between the cells. If one were to focus on the drop of media hanging from the inverted coverslip containing the cell preparations it was possible to find motile, morphologically intact sporozoites. These observations tend to argue against a harmful extracellular effect on the sporozoites but still leave the possibility of increased intracellular digestion as a result of contact with some extracellular factor.

In the third place, the difference in the mean percent infected cells of the various experimental groups could reflect a difference in the rate of removal of the parasite from the area of inflammation. The reports of studies on parenteral inoculations of coccidia in chickens (Davies and

Joyner, 1962, Sharma, 1964, Long and Rose, 1965) indicate that sporozoites introduced intraperitoneally produce infections in the cecum which give rise to oocysts by the seventh day. Although it has not been studied, the macrophage could be involved in transporting the parasite directly to the cecum or via the circulatory system. Challey and Burns (1959) report that macrophages in the lamina propria of the ceca, after having engulfed sporozoites, carry this invasive stage back towards the lumen of the digestive tract. The reason for carrying the sporozoites to the lumen is not known but the authors suggest that this action might be an attempt by the macrophage to clear the area of parasites and thus may be an attempt at protecting the host. A similar phenomenon could be occurring in the studies reported here. The lowered number of infected cells in some groups may indicate an increased ability of those cells to clear the peritoneal cavity of parasites and to transport them to the blood stream or other areas in the host where they would be subjected to others of the hosts protective mechanisms.

In the fourth place, the differences in the mean percent infected cells of the various experimental groups may reflect a difference in the ability of the cells to engulf the sporozoites. The possibility of a cell bound antibody in attracting the sporozoite to the phagocyte may be involved. On the other hand, it may be that the cells are invaded by the

sporozoites and the mean percent infected cells in the groups reflects the ability of cells to become parasitized by the sporozoites.

In the fifth place, the lower number of observed infected cells in the groups may not actually indicate that fewer cells are infected but that the sporozoites, once within the cell, are difficult to distinguish and in some cases, cannot be distinguished. This may reflect an increased capability of the cells to effectively deal with the engulfed parasite, possibly digesting it. Without exception, the majority of engulfed sporozoites within the cells from those groups with lower means were indeed difficult to distinguish and were morphologically distorted. In some cases only the refractile globule of the sporozoites could be distinguished. At the other extreme, the majority of the sporozoites found in macrophages from normal chickens with no serum treatment were quite easily seen with regular light and phase optics and appeared as morphologically intact as the free sporozoites. Occasionally, a distinct sporozoite which did not appear to be harmed by the intracellular environment was found in a group in which the majority of the intracellular sporozoites appeared in various stages of destruction. This may reflect a heterogeneity of competence to handle the parasite among the population of cells. Such a heterogeneity among immunologically competent cells has been described and mentioned

earlier in this paper. The finding of apparently unharmed sporozoites in groups with low mean percent infected cells may also reflect the time at which the sporozoite entered the cell during the reaction period. Thus, a sporozoite engulfed late in the incubation period would presumably have a more normal appearance than a sporozoite that entered a cell early in the incubation period. Another aspect of the time sequence lends support to this fifth explanation. In those groups of chickens with low mean percent infected cells, the infected cells were generally found fairly early in the cell count of each chicken of these groups. Thus, when 500 cells were counted, the infected cells were most often found in the first two to three hundred cells counted.

Lending important support to the cytopepsis theory in explaining the differences in the number of infected cells were the results obtained when the cells were in contact with sporozoites for a total of 30 minutes. Whereas there was no statistical evidence to show a true difference in mean percent infected macrophages between normal and immune chickens in the 15 minute incubation groups, 30 minute incubation times, under identical conditions, did result in the demonstration of statistically fewer infected immune cells than normal cells. The variance in the number of both normal and immune infected macrophages was less than one. A statistical analysis of the percentage of infected cells derived from

normal birds within fifteen minutes incubation as compared with similar normal cells incubated with sporozoites for thirty minutes showed that there had actually been no change in the mean percent infected cells. This may be a result of the high variance associated with the group with fifteen minutes incubation. A similar lack of statistical significance exists when the corresponding groups of immune cells were analyzed.

There is no reason to think that only one of these hypotheses is involved. Sorkin (1963) discussed the role of cytophilic antibodies in serum which, in vitro, but not necessarily in vivo, become attached to cells which are thus prepared for specific interaction or adsorption with an antigen. These antibodies are not identical with the main body of precipitating antibody to the antigen in the same serum. They fix to all cells tested, with the exception of erythrocytes, including granulocytes of peritoneal exudates, macrophages, and cells derived from popliteal lymph nodes. Such cytophilic or cell bound antibodies may act in immunological recognition and when they become attached to an immunologically competent cell, the specific antigen is attracted to that cell. In the case of the experiments reported here, such a mechanism might be involved in those groups which were treated with sera. The attraction of sporozoites to the cells involved might make them more avail-

able for phagocytosis and they may then be digested in cells which may be better capable of breaking them down. Indeed, on several occasions sporozoites were seen attached by one end to macrophages but this was not a common occurrence and sporozoites which were apparently unaffected were seen moving around a macrophage which contained a sporozoite.

Although all these hypotheses could be used in explaining differences in the numbers of infected cells among the experimental groups, the interpretations involving the degranulated heterophils or "ring cells" imposes other problems involving the degranulation process itself. One might ask if the process is a natural phenomenon or if the loss of granules is a result of manipulating the cells. In a personal conversation, Dr. Alfred Lucas of the United States Department of Agriculture Avian Anatomy Project, Michigan State University, implied that it was not a technique artifact. The granules, in a sense, must be gotten rid of in order to make room in the cell for an object the size of a coccidian sporozoite. In their studies of exo-erythrocytic stages, Huff and Coulston (1944) report that all sporozoites of Plasmodium gallinarium were in degranulated heterophils only and not in intact heterophils. These workers report that the sporozoites in these cells were all destroyed. Hewitt (Lucas, 1961) attributes the loss of granules to degeneration or phagocytosis. Lucas (personal

communication) feels that the presence of sporozoites within these degranulated heterophils indicates they are capable of phagocytosis and are therefore not degenerating cells. It would appear then that degranulation of heterophils in this study was not necessarily the result of techniques of cell manipulation.

The cause of this degranulation is not known. There appears to be no particular pattern in the number of degranulated heterophils in the group and the number of ring cells varied widely in the various groups. A fixed and stained preparation appears to have more ring cells than does a living preparation of the exudate. That degranulation occurs upon contact with the inflammatory agent, balanced salt solution in this case, appears not to be the rule since many intact heterophils were present in all exudates. Contact with a phagocytizable particle such as a bacterium or a sporozoite may be required but many ring cells were found which appeared to be devoid of any foreign material.

The environmental nature of the vacuole which remains after degranulation is also unknown. If the granules are lysosomal in nature, their rupture may result in a vacuole filled with cytopeptic enzymes which would probably result in the destruction of any organism which might be engulfed. The whole problem of interpreting the results obtained in this study involving the ring cells can probably best be

noted by observing the large variances in infected ring cells among the groups. A high percentage of infected ring cells may not reflect the cells reaction to a sporozoite or its ability to deal with the intracellular sporozoite but merely reflect the rate of degranulation. In one immune bird, for example, only ten ring cells were found but six of these contained sporozoites giving a large percent of infection. There were many intact heterophils in the same exudate which presumably had the potential of becoming infected if degranulation were to occur.

Hirsch (1965) describes the rupture of polymorphonuclear leucocyte lysosomes in the region of engulfed bacteria. He refers to this process as degranulation and indicates that it results in the release of enzymes from the lysosomes. These enzymes are responsible for the intracellular destruction of engulfed bacteria. It is important to note that degranulation as described by Hirsch (1965) and degranulation as described in this paper are not necessarily the same phenomena. Hirsch does not mention degranulation in the absence of phagocytosis. In the studies reported in this paper, degranulation of heterophils occurred without any evident phagocytosis.

Based on the arguments put forth above, experimental results, and observations on infected cells, the differences in the numbers of infected macrophages from the experimental

groups in this study can probably best be interpreted as differences in the abilities of the cells to break down the intracellular sporozoite. On this basis, macrophages derived from immune birds (immune macrophages) are not any more capable of destroying sporozoites of Eimeria tenella than are macrophages obtained from peritoneal exudates of susceptible birds (normal macrophages) if only fifteen minutes contact with the sporozoites is allowed. If, however, a period of thirty minutes contact between macrophages and sporozoites is allowed, immune macrophages are significantly better able to cope with an intracellular sporozoite than are normal macrophages, as shown by significantly lowered percents of detectable infected cells.

At 15 minutes incubation, normal serum enhances the ability of normal macrophages to break down the intracellular sporozoite. Immune serum has no effect on normal macrophages as shown by the absence of statistical significance in the mean percent infected cells between normal macrophages and normal macrophages treated with immune serum. Normal serum did not enhance the ability of immune macrophages to destroy sporozoites but immune macrophages treated with immune serum showed greater destructive activities against sporozoites as compared with untreated immune macrophages. Immune macrophages treated with immune serum were better able to break down sporozoites of E. tenella than were normal or immune

macrophages without serum treatment, than were normal macrophages receiving either immune serum or normal serum treatment, or immune macrophages treated with normal serum.

In summary, based on the mean percent infected cells of the various experimental groups, analyzed with the "t" test at a 2.5 percent level of significance, macrophages from the peritoneal exudates of birds resistant to Eimeria tenella infections are not better able to deal with intracellular sporozoites than are normal macrophages unless they are in contact with the sporozoites for thirty minutes or are treated with either immune or normal serum before engulfing the sporozoites. At fifteen minutes of incubation, immune serum enhanced the digestive ability of immune macrophages but not macrophages derived from susceptible chickens.

A statistical analysis of the variances of the mean percent infected macrophages of the various experimental groups using the F test also shows some interesting trends. The treatment of either normal or immune macrophages with either normal or immune serum greatly reduces the variances when compared with either cells without serum treatment. Furthermore, the reduction in variance is the same with both cells no matter whether immune or normal serum is used. This might indicate that both immune and normal macrophages engulf the sporozoites in a random fashion but that the addition of either serum adds some specificity to their task, possibly

reflecting the appearance of some attracting factor such as a cytophilic antibody.

With all the difficulties encountered in interpreting the results involving degranulated heterophils being kept in mind, a few remarks can be made concerning these cells. There was no statistically significant differences in the mean percent infected ring cells derived from immune chickens (immune ring cells) compared with the mean percent infected ring cells derived from normal chickens (normal ring cells) at either 15 or 30 minutes incubation.

Normal serum treatment of both immune and normal ring cells is associated with a significant decrease in the number of infected cells compared with the same cells not treated with serum. There were statistically fewer infected normal ring cells treated with immune serum but there was no difference in the number of infected immune cells treated with immune serum compared with the number of infected immune cells not treated with immune serum.

In summary, based on the mean percent infected degranulated heterophil cells of the various experimental groups, analyzed with the "t" test at 2.5 percent level of significance, ring cells from the peritoneal exudates of birds resistant to Eimeria tenella contain the same number of infected cells as do those from susceptible birds. The number of infected ring cells from both immune and normal birds

treated with normal serum is less than the number in untreated immune or normal cells. Immune serum appears to have no effect on immune cells but does decrease the number of infected normal cells.

As reviewed earlier in this paper, several studies have been carried out which have shown that the immune mechanism of chickens against Eimeria tenella is effective after the sporozoite has left the lumen of the digestive tract but before any first generation merozoites are produced (Tyzzer, Theiler, and Jones, 1932, Pierce, Long, and Horton-Smith, 1962, Horton-Smith, Long, and Pierce, 1963). Challey and Burns (1959) have shown that macrophages of the lamina propria are active at this time in transporting sporozoites to the deep glands of the ceca where they undergo further development. The studies reported in this paper suggest that the macrophages of immune birds may be an active participant in the immune mechanism. Without the presence of serum of any source, fewer macrophages from the peritoneal exudates of immune chickens were demonstrated to contain sporozoites than were similar macrophages derived from normal chickens. The sporozoites in these infected immune cells were for the most part difficult to distinguish and had lost some morphological integrity. The appearance of the sporozoites within these cells has led to the hypothesis that the cells of immune chickens are more capable of destroying the sporozoites

than are those of normal chickens. Contact with an immune macrophage, however, may only be a step in a chain of events leading to the destruction of the parasite. The macrophage may alter the molecular makeup of the cell membrane of the sporozoite in such a way as to render it incapable of responding to a host stimulus regulating further development in the cecal cell. Thus, it may not be necessary for the sporozoite to be within the macrophage environment for a prolonged length of time. This could explain the reported failure of first generation merozoites to develop in immune chickens experimentally inoculated with sporozoites. Contact with a macrophage in an immune chicken may not destroy the sporozoite but render incapable of responding to possible host stimuli regulating the development of schizonts from sporozoites.

Burns and Challey (1959) and Horton-Smith, Beattie, and Long (1961) have shown that the resistance to Eimeria tenella may be transferred from an infected cecum to its ligated partner which had not been in contact with the parasite. Failure to passively immunize susceptible chickens with immune serum has indicated that a humoral mechanism is not directly involved in this transfer of resistance. The results of the studies reported in this paper offer support to a possible explanation put forth in one of the papers (Horton-Smith, Beattie, and Long, 1961). Since a connection

between the two ceca was maintained via the circulatory system, lymphoid cells may be involved in the transfer. Rebuck, Boyd, and Riddle (1960) have shown the transformation of lymphocytes to macrophages in areas of inflammation. Thus, macrophages or potential macrophages could be carried from the infected cecum to the uninfected cecum and transfer a cell based resistance. The demonstration that immune serum apparently enhances the ability of macrophages to deal with the parasite could also be involved in explaining the transfer of resistance to a ligated cecum. Macrophages transported in the blood stream would come in contact with immune serum and lymphoid cells which might have the potential of differentiating into macrophages would also encounter the effects of serum in the chicken which has been infected by Eimeria tenella.

These studies also offer a clue as to the failure of all attempts to passively immunize susceptible chickens with serum from resistant chickens which reacts, in vitro, with the various stages of the life cycle. The results showed that immune serum had no significant effect on the mean percent of infected normal macrophages. Immune serum had an effect only on macrophages from immune birds. Thus, if a substantial part of the mechanism of immunity to Eimeria tenella resides in these macrophages, the addition of immune serum to susceptible chickens would not be of any benefit.

Normal serum had an effect only on normal macrophages and did not significantly alter the number of macrophages from immune chickens which became infected.

The immunological aspects of the studies reported here are also in line with the results of recent reports on studies of immunity to coccidiosis in bursectomized chickens. These studies have implicated the role of the cellular factors associated with delayed hypersensitivity and homograft rejections in the mechanism of this immunity. Lymphocytes are generally implicated in these phenomena and these cells, as mentioned earlier, have the ability to be transformed into macrophages. Furthermore, Cooper, Peterson, South, and Good (1966) have indicated that the activity of macrophages in taking up colloidal gold is not affected by removal of the bursa of Fabricius and macrophages could then remain active in bursectomized chickens.

The complete significance of the results of these experiments will not be known without the benefit of future studies. Chief among these might be a study of the degranulation of heterophils concerning the mechanism of degranulation, the fate of the granules, and the nature of the intracellular environment following degranulation. Secondly, a study of the macrophages involved, their origin and ultimate fate, would be of value. The macrophages utilized in these experiments may not be similar to those of the cecal lamina propria

and cecal macrophages may react with sporozoites of Eimeria tenella in an entirely different manner. The fate of the intracellular sporozoite needs to be determined. This may possibly be facilitated by the use of some marker which would be visible with florescence microscopy or with some type of radioactive label. Tests which would control the effects of the coelomic environment and the potential role of cytophilic antibody in interpreting these results should be carried out. The intracellular sporozoite also might lend itself to study with electron microscopy. With such a technique, membrane studies could be carried out which might not only clarify the role of the macrophage in coccidiosis infections but may offer clues as to host cell coccidia relationships in general. Lastly, the sporozoite-macrophage relationship should be studied as an aid in the successful in vitro cultivation of Eimeria tenella. Recent reports (Patton, 1965), Strout, Solis, Smith, and Dunlop, 1965) have indicated that cell culture techniques have not met with complete success in this venture. The failure of coccidia to carry out their life cycle in large numbers in cell culture may be due to the failure of the sporozoite to encounter the intracellular environment of the macrophage. Perhaps acquisition of factors from the macrophage or the partial breakdown of the sporozoite cell membrane by the macrophage is necessary for the parasite to respond to factors from the cecal gland epithelium to permit further growth and development.

# SUMMARY AND CONCLUSIONS


Summary:

A series of experiments was carried out to investigate the possible role of phagocytic cells in the mechanism of the immunity to Eimeria tenella which develops in chickens following their recovery from cecal coccidiosis. Peritoneal exudates were artificially stimulated with injections of Hanks' balanced salt solution into the coelom of normal chickens and chickens hyperimmunized against E. tenella. In some cases, serum derived from normal chickens or chickens immunized against E. tenella was injected intraperitoneally during the development of the exudate to determine the effect of serum on the cells of the exudate. Forty-eight hours after the first BSS injection, sporozoites of E. tenella were injected intraperitoneally and after 15 or 30 minutes of incubation the cells were harvested. The percentage of exudate cells containing sporozoites was determined by microscopic examination.

Of the several cell types which could be identified in the peritoneal exudates, only macrophages and heterophil leucocytes which had lost their rod shaped granules were observed to contain sporozoites. Based on the mean percent of infected cells, there was no difference in the number of

immune macrophages containing sporozoites compared to normal macrophages at 15 minutes of incubation. At the same incubation time, significantly fewer immune cells treated with immune serum contained sporozoites compared to untreated normal macrophages or untreated immune macrophages; but there was no difference in the number of infected normal macrophages treated with immune serum compared with untreated normal cells. There was no difference in the number of infected immune cells treated with normal serum compared with untreated immune macrophages but significantly fewer normal macrophages treated with normal serum were found to contain sporozoites when compared with untreated normal macrophages. If 30 minutes incubation time was allowed, significantly fewer immune macrophages than normal macrophages were found to contain sporozoites. In all groups of chickens which were found to have a lowered mean percent of infected macrophages, the intracellular sporozoites were difficult to distinguish within the cytoplasm and appeared, based on morphology, to be degenerating. It is hypothesized that the differences in the mean percent of infected cells among the experimental groups can be explained primarily by an increased ability of immune macrophages and immune macrophages treated with immune serum to break down the intracellular sporozoites.

There was no significant difference in the mean percent of infected ring cells derived from immune chickens compared

with ring cells derived from normal chickens at either times of incubation. There were fewer normal and immune ring cells treated with normal serum containing sporozoites than the respective untreated cells. Immune serum did not significantly alter the mean percent infected ring cells from immune chickens but significantly fewer normal ring cells treated with immune serum were found to contain sporozoites than untreated normal ring cells. Because of a lack of understanding of the degranulation phenomenon in heterophil leucocytes, it is difficult to interpret these results.

The finding that immune macrophages, with or without the presence of immune serum are apparently better able to destroy sporozoites offers an explanation for certain immunological phenomena associated with cecal coccidiosis in chickens. Since macrophages have been observed to transport sporozoites in the early stages of the life cycle, these results offer an explanation as to how an immune chicken is protected against challenge infections and explains why first generation schizonts fail to develop in immune chickens. The failure of immune serum to alter the cytopeptic activities of normal macrophages might explain the failure to passively immunize normal chickens using immune serum. The potential role of macrophages in the mechanism of immunity helps to explain the transfer of immunity from a ligated cecum to its non-ligated partner.

Suggestions for further research are given including studies to follow the intracellular breakdown of sporozoites and of the process of degranulation of heterophil leucocytes.

Conclusions:

The present study establishes the macrophage and heterophil leucocyte as potential mediators in the mechanism of the resistance of chickens to reinfection by Eimeria tenella. More efficient clearing of sporozoites from the area of inflammation is indicated by significantly fewer macrophages derived from immune chickens than macrophages derived from susceptible chickens can be observed to contain sporozoites. This effect was enhanced by treatment of the cells with serum derived from immune birds. The difficulty in observing the sporozoites in immune cells and their distorted morphologic appearance in these cells indicates a greater ability on the part of the immune macrophage to destroy the parasite. Failure of macrophages in immune chickens to pick up sporozoites, a second possible interpretation of the data reported here, might also be a significant factor in resistance. In this case, sporozoites are unprotected and more susceptible to other immune mechanisms of the resistant chicken.

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