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LOCAL IMMUNE RESPONSE TO *EIMERIA TENELLA* INFECTION

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ABSTRACT: The present study was undertaken to investigate the local immune response which is produced in the caecal lymphoid tissue of chickens infected with *Eimeria tenella* at 1 day of age. Both flow cytometric analysis and immunohistochemical staining on frozen sections were carried out using specific monoclonal antibodies against chicken leukocytes, monocytes/macrophages, cells expressing CD4 and CD8 antigens, IL2 containing cells, and Ig synthesizing cells. In correlation, anti-*E. tenella* immunoglobulin synthesizing cells were also studied. In the caecal lymphoid tissue of parasitized chickens, we could observe an increase in both macrophages (68.1$^+$ cells) and CD4$^+$ T cells from 24 h postinfection onwards, whereas CD8$^+$ T cells significantly increased on day 5. No such increase could be observed in uninfected birds. The subsequent appearance of specific antibody-containing cells was detected from day 5 onwards.

KEY WORDS: Sporozoa, *Eimeria tenella*, B cell, CD4 cell, CD8 cell, IL2, macrophages.

INTRODUCTION

Coccidiosis, which is caused by obligate, intracellular, protozoan parasites belonging to several species of the genus *Eimeria*, is a major problem for the poultry industry. Currently, the disease, which is controlled by the use of prophylactic chemotherapy, costs the broiler chicken industry wordwide approximately two billion dollars per annum (BHOGAL *et al.*, 1992). The difficulties and expenses involved in the development of new chemoprophylactic agents, drug resistance and residue problems, justify the development of immunological prophylaxis. Avian coccidiosis vaccine gives protection against the pathogenic effects of coccidial infection, but it does not induce an absolute immunity to infection. Thus, the vaccine for coccidiosis has the disadvantage that, unless the dosing of very large numbers of birds is carefully controlled, there is a possibility that the parasites will cause disease as they recycle through animals which have been insufficiently immunized. As a consequence, the immunological aspects of the host-parasite relationship are currently receiving more attention. Numerous reports have considered a multitude of questions related to the protective immune response, which is induced during an experimental *Eimeria* infection in 4-6 week-old chickens. These include the type of effector cell involved in resistance (LILLEHOJ & BACON, 1991), the cells involved in both the transport of the parasite (LAWN & ROSE, 1982) and the presentation of the antigen (DEL CACHO *et al.*, 1993a), as well as the protective antigens recognized (DANFORTH & AUGUSTINE, 1990). However, chickens are vaccinated against coccidia by one week of age. This makes it convenient to increase our knowledge regarding the immune mechanisms involved in the gut immune response shortly after hatch. However, little, if any, information has been published concerning the role of the immunocompetent cells in *Eimeria tenella* infection during the first three weeks of life. On the other hand, previous studies have reported an age-related maturation of the gut-associated lymphoid tissues (BEFUS *et al.*, 1980; JEURISSEN, JANSE & KOCH, 1988a; LILLEHOJ & CHUNG, 1992). Therefore, one may assume that the capability of chicks during the early days of life to evoke an immune response is different from that of 4-6-week old chickens. These considerations have prompted us to study the dynamic changes that the cell populations involved in the mucosal immune response to coccidia undergo, following an *E. tenella* infection at one day of age. For this purpose we have combined both flow cytometry and *in situ* immunohistochemistry, using a panel of chicken-lymphocyte-specific monoclonal antibodies.

MATERIAL AND METHODS

Animals: Male and female one-day-old Leghorn chickens were obtained from a commercial hatchery and reared and maintained in elevated wire-floored coccidia-free cages until used in the experiments. Periodical faecal examinations for oocysts were made to assure freedom from infection prior to experimental use. No coccidial or antibiotic compounds were present in the food. The chickens were killed by cervical dislocation.

Parasite: An *Eimeria tenella* line was originally obtained from Merk, Sharp and Dome (Madrid, Spain). Chickens were infected with sporulated oocysts, stored in 2.5% potassium bichromate solution for less than 4 weeks, by oral innoculation into the crop. The

methods used for the maintenance and preparation of oocysts for inoculation have been described in the literature (LONG *et al.*, 1976).

Experimental design: A total of one hundred and twenty 1-day-old chickens was used. The birds were randomized into 2 groups of 60 chickens with equal mean weight. The chicks in the first group received a single dose of 100 oocysts 24 hours after hatch (day 0). The second group (control group) consisted of uninfected chickens. Ten chickens from each of the groups were killed and the distal and proximal (containing the caecal tonsil and lymphoid nodules) areas of the caeca were removed on days 1, 3, 5, 7, 14, and 21 postinfection (p.i.). The right distal and proximal caecal samples from each chicken were used for flow cytometric studies and the left caecal samples were routinely processed for light microscopy examination. For this purpose samples of the caecal tonsils and lymphoid nodules in the proximal caecum (DEL CACHO *et al.*, 1993b), as well as samples of the distal caecum were either frozen in liquid nitrogen or fixed in Bouin's liquid for 90 minutes.

Flow cytometric analysis: Cell suspensions were obtained from the mucosa of both the distal and proximal (caecal tonsil and mucosa associated lymphoid nodules) caeca by passing the tissues through a wire mesh, followed by centrifugation over Ficoll-Hypaque gradient in order to remove dead cells, red blood cells, and epithelial cells. Final cell viability, determined by trypan blue exclusion, was >90% for all tissue samples. Cell suspensions from the mucosae of the distal and proximal caecum were prepared in ice-cold phosphate-buffered saline (PBS), pH 7,2, supplemented with 3% newborn calf serum (NCS) and 0,01% NaN_3 (PBS-NCS). The leukocytes ($2x10^6$) were incubated on ice for 30 min with 0,03 ml of the following monoclonal antibodies: CVI-ChNL-68.1 (specific for chicken monocytes and macrophages) (JEURISSEN *et al.*, 1988b), and anti-CD4 and -CD8 (CHAN *et al.*, 1988). After washes, a second incubation was carried out with 1:100 dilution of fluorescein-conjugated rabbit anti-mouse immunoglobulin (Dako Laboratories, Denmark) for 30 min. Cell suspensions were analysed using a FACScan (Beckton Dickinson, Mountain View, Ca). Dead cells were excluded by propidium iodine staining. A total of 5000 gated leukocytes, on the basis of forward and side light scatter, were analysed for each sample, using Lysis II software (Dako Laboratories).

Immunobistochemistry: Cryostat sections (8 µm thick) of frozen caecal samples were picked up on glass slides and stored at room temperature. The slides were fixed for 10 min in 100% acetone and air-dried. Endogenous peroxidase was inactivated with a solution of 0,5% hydrogen peroxide in ethanol for 30 min at room temperature. The slides were then washed in buffer and incubated in normal swine serum for 10 min, followed by incubation with CVI-HIS-C7 (chicken leukocytes) (JEURISSEN *et al.*, 1988), or with either of the above mentioned monoclonal antibodies, 68.1, anti-CD4 and -CD8. The sections were incubated with a biotinylated anti-mouse antiserum (Vector Laboratories, Burlingame, Ca) for 30 min. Avidin-biotin peroxidase complex (Vector) was applied for 45 min, and the peroxidase reaction developed with diaminobenzidine (DAB) and hydrogen peroxide solution (20 mg DAB in 100 ml 0.05 M TRIS-HCl buffer pH 7.6 containing 0,005% H_2O_2) for 5 min.

Paraffin sections (5-7 µm) of Bouin's liquid-fixed tissues were used to detect IgG, IgM, and IgA synthesizing cells at the light microscopic level. Deparaffinized sections were rinsed in PBS (pH 7,2), and endogenous peroxidase was inactivated as described above. The slides were incubated in normal swine serum for 10 min, followed by incubation with either antichicken-IgG, -IgM, or -IgA (INC Immune-Biologicals, Leslie, Il) as primary antibody at a 1:10000 dilution, each for 18 h at 4° C. Then, paraffin sections were treated following the procedure mentioned above, which was applied to cryostat sections. The slides were counterstained with hematoxylin and mounted.

IL2 mRNA containing cells were detected by *in situ* hybridization using a digoxigenin-labelled double stranded DNA probe (168 bp) obtained by PCR from rat IL2 mRNA.

Serial frozen tissue sections were cut at three levels from each sample for *in situ* hybridization and were immediately fixed in 4% paraformaldehyde pH 7,2 for 60 minutes. The sections were permeabilized with 0,1 triton X-100 and dehydrated in graded ethanol.

The denatured labeled DNA was mixed with 50% formamide, 0,6M NaCl, 50 mM Tris, 5 mm EDTA, 10 mg/ml denatured herring sperm DNA, 10 mg/ml tRNA.

Twenty-five ml of hybridization solution was applied to each slide. Hybridization was carried out for 18 h at 37° C in a humidified chamber. Endogenous peroxidase was inactivated with a solution of 0,5% hydrogen peroxide in ethanol for 30 minutes at room temperature. The slides were incubated in normal swine serum for 10 minutes, followed by incubation with an anti-digoxigenine monoclonal antibody which was produced in sheep. The sections were incubated with a biotinylated anti-sheep antiserum (Vector Laboratories, Burlingame, Ca) for 30 minutes and the peroxidase reaction developed as described above.

Detection of anti *E. tenella* antibody producing cells: In order to enumerate the cells secreting anti-E.tenella antibodies of the IgG, IgA, and IgM isotypes, an agarless monolayer haemolytic plaque technique, as modified for the chicken system by MULLER, SATO & GLICK (1971) was employed. The reaction mixture consisted of 0,8 ml of 3% antigen coated sheep red blood cells (SRBC) in Hank's balanced salt solution (HBSS), 0,1 ml of fresh normal chicken serum as a complement source, 0,1 ml of caecum cell suspension ($5,0\times10^6$ cells/ml) in HBSS, and 300 µl of antichicken-IgG or -IgA. A measured amount (14,5 µl) of reaction mixture was dropped onto a modified microscope slide (JEURISSEN *et al.*, 1988) and incubated at 37° C for 30-40 min. The results were expressed as the number of plaque-forming cells (PFC) per 10^6 lymphoid cells. The antigen used to coat the SRBC was obtained as described by VERVELDE, VERMEULEN & JEURISSEN (1992). Briefly, sporulated oocysts of *E. tenella* were suspended in a solution containing phenylmethylsulfonyl fluoride and broken by shaking them with glass beads for 5 min. The antigen was obtained by sonication of this suspension on ice. The suspension was then incubated with 0,1% TX-100 for 1 h on ice and clarified by centrifugation at 3500 g. The antigen was coupled to SRBC using a solution of chromic chloride in 0,15 M NaCl at a concentration of 10 mg/100 ml.

Statistical analysis: Data were analysed by factorial analysis of variance. Differences between means for multiple comparison were assessed by Duncan's New Multiple Range Test with statistical significance inferred at $P < 0,005$.

RESULTS

Flow cytometric analyses of the lymphoid tissue from the proximal caecum

The data referring to the caecal tonsil and lymphoid nodules in the proximal caecum are shown in Fig. 1. In control chickens, from day 1 to 3 weeks of age, the percentage of $CD4^+$, $CD8^+$, and 68.1^+ cells increased in the caecal tonsil and lymphoid nodules in the proximal caeca (Fig. 1). $CD4^+$ cells gradually increased after hatch, and on day 21 they made up 15,3% of the total cells (Fig. 1). The percentages of $CD8^+$ cells, which increased

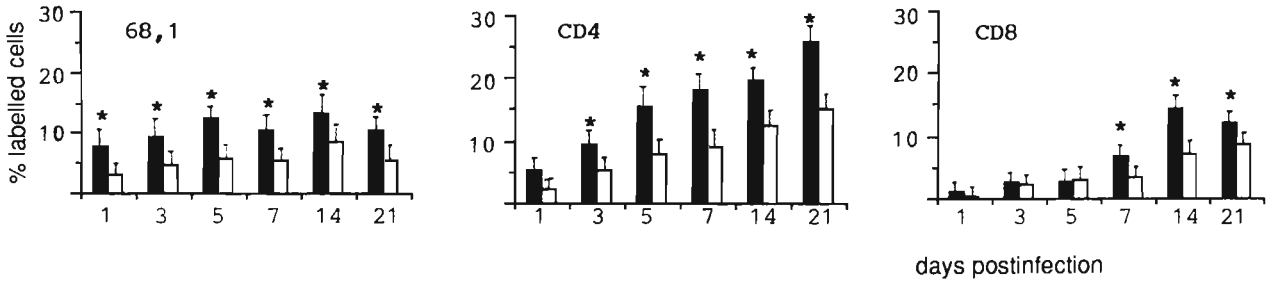

Fig. 1.– Percentage of 68.1$^+$, CD4$^+$, and CD8$^+$ cells in the lymphoid tissue of the proximal caeca of *Eimeria*-infected (■) and control chickens (□). Each bar represents the mean ± s.d. Significant differences between the groups on each day are indicated as * ($P<0.005$).

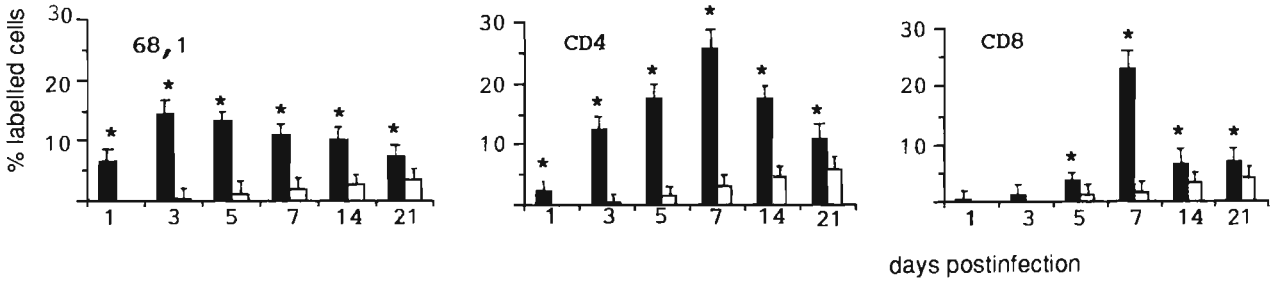

Fig. 2.– Percentage of 68.1$^+$, CD4$^+$, and CD8$^+$ cells in the lymphoid tissue of the distal caeca of *Eimeria*-infected (■) and control chickens (□). Each bar represents the mean ± s.d. Significant diferences between both groups are marked as * ($P<0.005$).

slowly during the first week of life, were lower than those of CD4$^+$ cells (Fig. 1).

In infected chickens, the increases in the percentages of the cell populations in the mucosa of the proximal caeca were parallel to those found in controls, but at all sampling times the numbers of lymphoid and non-lymphoid cells were higher in infected chickens than those in controls (Fig. 1). The percentage of CD4$^+$ cells increased with time p.i., reaching a maximun value on day 21. No significant differences in the proportion of CD8$^+$ cells were found between parasitized and control chickens until day 7. On day 7 the values of CD8$^+$ cells in parasitized chickens significantly increased, reaching a maximum on day 14 with a subsequent decline to day 21, whereas the number of CD8$^+$ cells in controls constantly increased until the end of the experiment on day 21.

Flow cytometric analyses of the lymphoid tissue from the distal caecum

The data referring to the distal caecum are presented in Fig. 2. In control chickens, the development of the lymphoid tissue in the distal caecum was significantly lower than that in the proximal region. Indeed, during the first week of life, there was an almost total absence of positive CD4, CD8, and 68.1 cells in the distal caeca of the control chickens (Fig. 2). What is more, twenty-one days after hatch, the percentage of lymphoid and non-lymphoid cells in the distal caeca was markedly lower than that found in the proximal region.

Significant differences between control and parasitized chickens in the proportion of 68.1$^+$ or CD4$^+$ cells occurred during the first week p.i. The proportion of both CD4$^+$ and 68.1$^+$ cells reached maximum values on day 7, gradually declining during the second and third weeks p.i. The percentages of CD8$^+$ cells did not show any significant variation with respect to the control values until day 5. The percentage of CD8$^+$ cells reached a peak on day 7, declining sharply thereafter.

Anti-*E. tenella* immunoglobulin synthesizing cells

The data referring to the cells capable of producing anti-*E. tenella* antibody in infected chickens are given in Fig. 3. No Ig-synthesizing cells were found before day 5 p.i. in the lymphoid tissue of either the distal or proximal caeca. The numbers of IgG- and IgM-producing cells did not significantly increase until day 5 p.i., and those of IgA-producing cells did not increase until day 7. On day 5, the predominantly produced isotype was IgM, but in the following days IgG-producing cells became the most frequent Ig-synthesizing cell, in both the proximal and the distal caecum. The number of IgM-producing cells was slightly higher than that of IgA-producing cells in both caecal regions, although the difference was more evident in the proximal caecum (Fig. 3). However, the total number of anti-*E. tenella* antibody producing cells was roughly ten times higher in the distal than in the proximal caecum (Fig. 3). Maximum numbers for either IgM-, IgG-, or IgA-producing cells occurred on day 21 p.i.

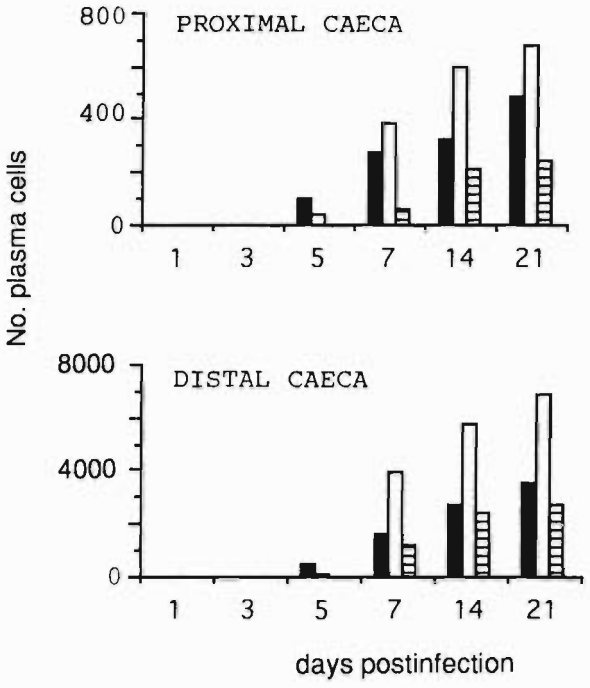

Fig. 3.– Number of anti-*E. tenella* antibody synthesizing cells in the distal and proximal caeca of chickens after *E. tenella* infection. (■) = IgM-synthesizing cells; (□) = IgG-synthesizing cells; (▤) = IgA-synthesizing cells. Each bar represents the mean ± s.d.

(Fig. 3). The uninfected chickens (controls) did not have cells which synthesized specific Igs against *E. tenella*.

Distribution of cells involved in the immune response to *E. tenella* in the caecum

Hardly any lymphoid tissue was observed in the distal caeca of the uninfected chickens, where no lymphoid follicles were seen (the lymphoid tissue in the caeca is mainly located in the proximal caeca). Therefore, almost no cells were found in the distal caeca stained with any of the antibodies used.

Twenty-four hours after infection, His-C7$^+$ cells, a few of which were T cells expressing either CD4 or CD8, were observed around the end of crypts as isolated aggregates scattered throughout the lamina propria of the distal caecum (Fig. 4A). On days 3 and 5 p.i., the schizonts were surrounded by numerous 68.1$^+$ cells (Fig. 4B) and a few mRNA IL2 containing cells (Fig. 4G), whereas CD8$^+$ and CD4$^+$ cells rarely appeared near the parasite (Fig. 4C,D). Also in the distal caecum, numerous CD8$^+$ cells were found in the submucosa close to blood vessels, whereas they were very limited in the mucosa (Fig. 4C). CD4$^+$ cells were abundant in the deep region of the lamina propria in the distal caecum close to the submucosa and also appeared in the submucosa in association with the blood vessels (Fig. 4D). By contrast, on day 7 CD4$^+$ cells were found accumulated around the crypts, where oocysts were observed within the epithelial cells lining the lumen (Fig. 4E).

In the lamina propria of the proximal caecum, aggregates of His-C7$^+$ cells were seen on days 3 and 5 p.i. Most of these positive cells were Ig containing-cells and CD4$^+$ cells. CD8$^+$, 68.1$^+$, and mRNA IL2 containing cells were also found in the above-mentioned cell aggregates. On day 7 p.i., in the distal caecum, Ig containing cells, CD8$^+$ cells (Fig. 4F), and IL2 containing cells infiltrated the lamina propria. There was a great density of CD8$^+$ cells around the crypts which were invaded by the parasite (Fig. 4F). Furthermore, CD8$^+$ cells were seen between enterocytes (Fig. 4F).

As early as 7 days p.i. parasitized chickens exhibited germinal centers deep in the lamina propria of both the proximal and the distal caecum. These germinal centers contained IgG-, IgA-, and IgM-producing cells. In control chickens, germinal centers did not appear until day 21. On days 14 and 21 p.i., the presence of occasional epithelial cells showing cytoplasmic immunostaining for either IgG or IgA was detected (Fig 4H).

DISCUSSION

The earliest event that the authors observed in the current results was the accumulation of His-C7$^+$ cells around the crypts of the distal caecum 24 h p.i., coupled with a limited number of CD4$^+$ and CD8$^+$ cells in this location. This is due to the fact that the invasion of the sporozoites causes the accumulation of inflammatory cells such as macrophages, neutrophils and mast cells. ROSE, WAKELIN & JOYSEY (1989) and JOYSEY, WAKELIN & ROSE (1988) have suggested that the inflammatory cells are not responsible for the immunity against *Eimeria*. Therefore, the concentration of inflammatory cells may be unimportant in the destruction of the parasite, although the inflammatory response is a significant event in coccidial infections (FERNANDO, 1982).

Our results indicate that the schizonts were observed at the same time as three cellular events: 1) the macrophages were very close to the schizonts and even surrounded the parasite; 2) the IL2 synthesizing cells increased in number; 3) the T cells expressing CD4 or CD8 antigens increased in number. The capability of the merozoite and schizont antigens to promote the proliferation of T lymphocytes is well-known (LILLEHOJ, 1986; MARTIN & LILLEHOJ, 1993). Three steps are necessary for T cell proliferation: a) the interaction of the Ia-bearing cells with the antigen; b) the release of IL1 from this cell and the response of a T cell to this factor; c) the release of IL2 from the cell mentioned in step b and the response of the potential effector T cell to IL2 (SCHNETZLER *et al.*, 1983). Therefore, it is likely that a relationship could exist between the three above-mentioned cellular events and the presence of schizonts of both first and second generation, the latter being a more immunogenic stage. The macrophages could process the merozoite and/or schizont antigens, present these antigens in association with MHC molecules to the T cells, and release IL1.

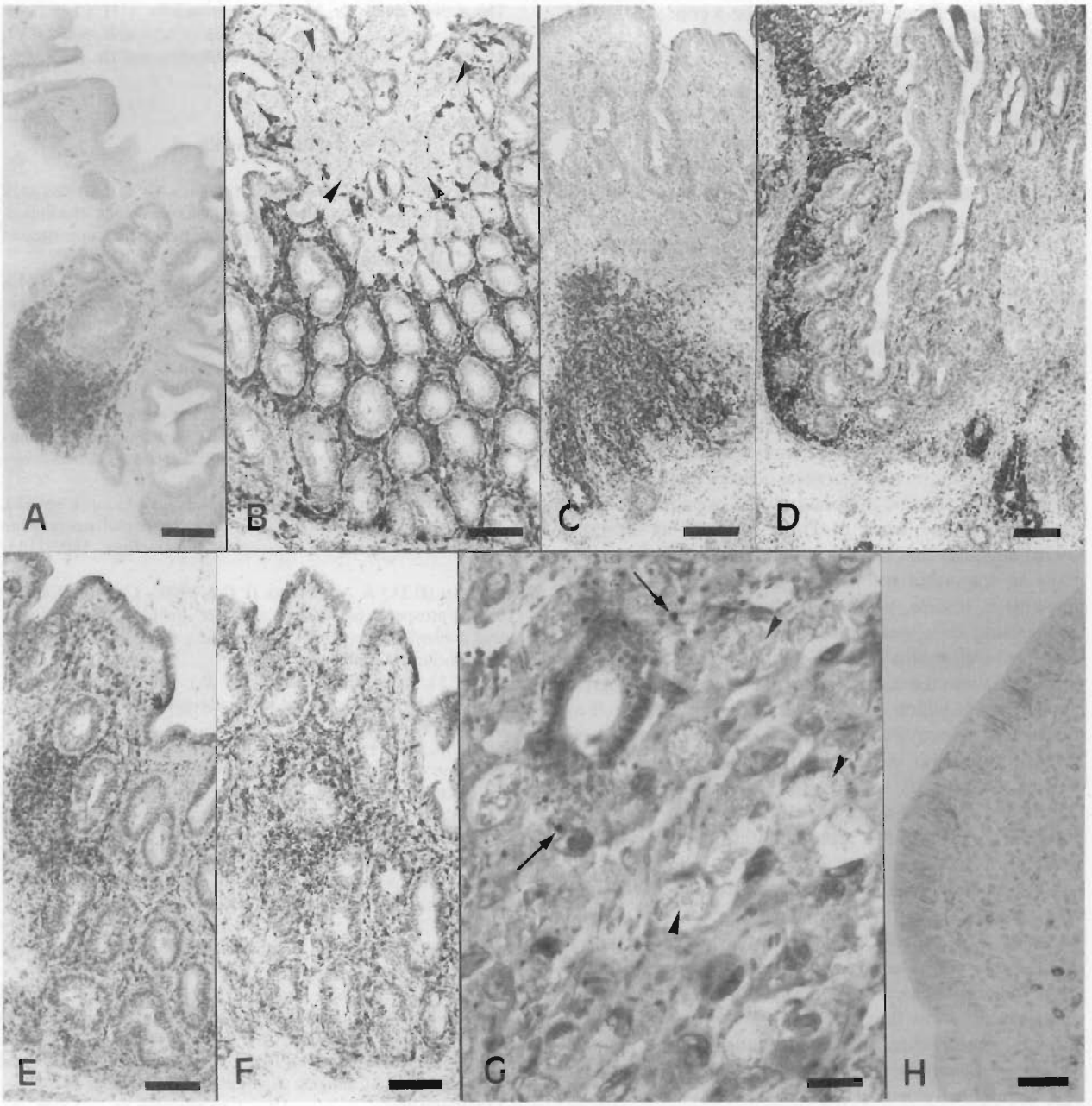

Fig. 4.– Distribution of positive cells in the distal caeca of chickens infected with 100 oocysts at 1 day of age. A) His-C7$^+$ cells 24 hours p.i.; B) 68.1$^+$ cells 3 days p.i.; schizonts shown by arrowheads; C) CD8$^+$ cells 5 days p.i.; D) CD4$^+$ cells 5 days p.i.; E) CD4$^+$ cells 7 days p.i.; F) CD8$^+$ cells 7 days p.i.; G) mRNA IL2 containing cells (arrows) 5 days p.i.; schizonts shown by arrowheads; H) IgG$^+$ cells 14 days p.i. Scale bars: A, B, C, D, E, F = 600 µm; G = 300 µm; H = 400 µm.

Those T cells that had recognized the antigens would release IL2.

The major function of IL2 is the activation of a variety of cells in the immune system, including helper T cells, cytotoxic T cells, B cells, macrophages, natural killer cells, and lymphokine-activated killer precursors. In addition to acting in a paracrine fashion, IL2 is also able to act as an autocrine growth factor. Autocrine stimulation enables rapid clonal expansion of antigen-activated cells (DELVES & ROITT, 1992). The finding that up to 7 days p.i., CD4$^+$ and CD8$^+$ cells were more numerous in the area where the schizonts were observed suggests that these T cells expressing CD8 and CD4 reached that area from the blood vessels in the submucosa. Thus, the increase in the numbers of CD8$^+$ or CD4$^+$ cells, which was observed in the distal caecum, may be a consequence of the accumulation of migrating cells from outside the lamina propria. However, in subsequent days the increase

in the number of these T cells could be a consequence of proliferation induced by IL2.

The maximum numbers of $CD4^+$ and $CD8^+$ cells were observed in the distal caecum on day 7 p.i. Besides, these cells appeared around the crypts, and IL2-releasing cells were detected among the cells expressing CD4 and CD8 antigens. Taken together, these findings indicate that, at this stage of infection, there could be a relationship between $CD4^+$ and $CD8^+$ cells, in the sense that CD4+ cells could influence cytotoxic T lymphocytes to induce the cytolytic activity of the CD8 cytotoxic T cells. This hypothesis is in accordance with the data reported by ROSE, JOYSEY & HESKETH (1988) on the role of CD4 cells in controlling infection with *Eimeria*.

Although the cell-mediated immune response plays a primary role in the protective immune response to coccidiosis (ROSE, 1982; LILLEHOJ & TROUT, 1993), defense against *Eimeria* is also due to humoral immunity. Indeed, we found appearance of germinal centers and increase in the number of specific antibody-producing cells on day 7 p.i. Although the work of DAVIS, PARRY & PORTER (1978) suggests that the intestinal secretory IgA system plays an important role in the protective immune response to *E. tenella*, we found a higher number of IgG-synthesizing cells than those synthesizing IgA. In addition, our results show that IgG is transported through the enterocytes into the caecal lumen. The IgG has biological characteristics which are not shared by the IgA, such as the capacity to activate the classical pathway C and an efficient localization in follicles (KLAUS *et al.*, 1980), as well as the capacity to activate the antibody-dependent cell-mediated cytotoxicity (ROITT, BROSTOFF & MALE, 1986). In the light of this report and through our findings, we suggest that, in the gut, the IgG may play an important role in anticoccidial immunity. This is in accordance with previos studies by DEL CACHO *et al.* (1993) and SHAWKY, SAIF & MCCORMICK (1994) who have reported that the IgG could have an important functional significance in the protection of mucosal surfaces.

Our results show that although the number of specific antibody-synthesizing cells increased after the parasite disappeared from the gut, the number of $CD4^+$ and $CD8^+$ cells dramatically decreased.

As the disappearance of the parasite means the absence of antigen to stimulate T cells, this result is in agreement with the observations of GRAY & MATZINGER (1992), who have reported that the maintenance of cytotoxic T-cell memory requires the continuous restimulation by antigen, but argues against the proposal of MULLBACHER (1994) that the longevity of T cells is all that is needed for the cytotoxic T-cell memory response to be maintained.

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