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Immunohistochemical Studies on S100 Reactivity in Chicks Experimentally Infected with *Eimeria tenella* I: The Localization of S100 Protein and Its Subunits α and β in Stages of *Eimeria tenella**

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Abstract: The expression of the calcium-binding protein S100 and its subunits α and β in the intracellular protozoon *Eimeria tenella* was investigated. For this purpose, anti-S100 whole molecule polyclonal antibody and anti-S100α and anti-S100β monoclonal antibodies were used on paraffin sections collected from the cecum, ileum, colon, rectum, and bursa of Fabricius of chicks experimentally infected with *E. tenella*. One hundred seventy-five 2- to 3-day-old specific pathogen-free chicks were used. The chicks were divided into experiment and control groups, to include 94 and 81 chicks, respectively. All the chicks in the experimental group received 10,000 spored oocytes in 0.5 ml inoculum in a single dose. The duration of the experiment was limited to 18 days. All 175 chicks were sacrificed by cervical dislocation between hours 1 and 432 following inoculation. The streptavidin-biotin complex method was used to detect the immunoreactivity of the antibodies used. The results revealed a positive immune reaction for the S100 whole molecule and for α and β subunits in the schizonts, microgametes, macrogametes, and oocytes of the *E. tenella* in the cecum, ileum, colon, rectum, and bursa of Fabricius. These results show that both α and β subunits of the S100 protein existed in all the endogenous phases of *E. tenella*. These proteins are likely to mediate the parasitic development of *E. tenella* within host cells.

Key Words: *Eimeria tenella*, S100 protein, immunohistochemistry, chicks

Civcivlerde Deneysel *Eimeria tenalla* Enfeksiyonunda S100 Reaktivitesi Üzerine Immunohistokimyasal Çalışmalar I: *Eimeria tenella*'da S100 Proteini ve α ile β Alt Ünitelerinin İmmunohistokimyasal Lokalizasyonu

Özet: Bu araştırmada, intrasellüler protozoon olan *Eimeria tenella*'da bir kalsiyum bağlayıcı protein olan S100 ve onun α ve β alt ünitelerinin ekspresyonu incelendi. Bu amaç için, *E. tenella* ile deneysel olarak enfekte edilen civcivlerin sekum, ileum, kolon, rektum ve bursa Fabricius'larından alınan parafin kesitlere anti-S100 whole molekül poliklonal antikoru ile anti-S100α ve anti-S100β monoklonal antikorları uygulandı. Araştırmada, 2-3 günlük 175 adet spesifik patojen free (SPF) civciv kullanıldı. Toplam 175 adet civciv, deney ve kontrol olmak üzere sırasıyla 94 ve 81 civciv içeren 2 gruba ayrıldı. Deney grubundaki tüm civcivlere, tek dozda 0,5 ml inokulum içerisinde 10.000 sporlu oosist verildi. Deney süresi 18 günle sınırlandırıldı. Toplam 175 civciv inokulasyondan sonraki 1. saat ile 432. saat arasında servikal dislokasyon yöntemiyle öldürüldü. Kullanılan antikorların immunoreaktivitelerini göstermek amacıyla Streptavidin-biotin-kompleks (StreptABC) yöntemi kullanıldı. Elde edilen sonuçlar, sekum, ileum, kolon, rektum ve bursa Fabricius'da, *E. tenella*'nın şizont, mikrogamet, makrogamet ve oosistlerinde S100 whole molekülü ve α ve β alt ünitelerinin her biri için pozitif immunoreaksiyon olduğunu gösterdi. Bu sonuçlar, S100 proteinin hem α hem de β alt ünitelerinin *E. tenella*'nın tüm endojen safhalarında bulunduğunu ortaya koymuştur. Bu proteinler, muhtemelen, *E. tenella*'nın konakçı hücreleri içerisindeki parazitik gelişimine aracılık etmektedir.

Anahtar Sözcükler: *Eimeria tenella*, S100 protein, immunohistokimya, civciv

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Introduction

S100 proteins belong to the EF-hand type acidic Ca^{2+} -binding protein superfamily and have a wide distribution in various tissues (1-6). S100 was isolated from the cattle brain by Moore in 1965 for the first time and this protein has for many years been thought to be specific to the nervous system (4). In addition, successive immunohistochemical and biochemical studies have shown that S100 protein exists in tissues other than the nervous system (7).

S100 protein consists of 2 subunits, S100α (S100A1) and S100β (S100B), which are expressed selectively by certain types of cells, found in homodimers (αα dimer known as S100ao or ββ dimer known as S100b) or heterodimers (αβ dimer known as S100a) (8,9). In mammals, S100B is most abundantly found in melanocytes, chondrocytes, dendritic cells, adipocytes, and in the glia cells in the central and peripheral nervous system (3-5), while S100A1 is mostly found in cardiomyocytes, slow-twitch skeletal muscle cells (10), and renal cells (11).

These proteins are responsible for regulation of intracellular events such as protein phosphorylation, certain enzyme activities, the dynamics of cytoskeleton component, transcription factors, Ca^{2+} homeostasis, and cell proliferation and differentiation (3,4). Protein phosphorylation is thought to be an important signaling mechanism in the development and differentiation of the parasitic protozoa, and that merozoites require a microtubule polymerization for cell invasion (12,13). Therefore, protozoa probably express S100 proteins. Nevertheless, in studies where immunohistochemical techniques were used, an S100-like protein was detected in the schizonts, macrogametes, and oocytes of *E. tenella* in the cecum (14), whereas it was detected in the macrogametes of *E. brunetti* in the ileum (15).

This study examined the expression and localization of S100 protein and its S100α and S100β subunits by ABC immunoperoxidase method in the endogenous forms of *E. tenella*, which develops in organs such as the cecum, ileum, colon, rectum, and bursa of Fabricius of the chicks experimentally infected with *E. tenella*.

Materials and Methods

Animals

One hundred seventy-five 2- to 3-day-old specific pathogen-free (SPF) chicks were used, which were obtained from the Manisa Poultry Animals Research and Vaccine Production Center. The chicks were kept under routine laboratory conditions without coccidiosis, and without food or drink limitation. No anticoccidial or antibiotic compounds were added to either the feed or water of the chicks.

Parasite

The *Eimeria tenella* strain (Et 97B) was obtained from Zaragoza University, Faculty of Veterinary Medicine, Zaragoza, Spain. The *E. tenella* oocytes were proliferated, isolated, and sporulated by standard procedures in the laboratory (16). The chicks were infected with sporulated oocytes by oral inoculation into the crop.

Experiment Design

The 175 chicks were divided into experiment and control groups, to include 94 and 81 chicks, respectively. All the chicks in the experimental group received 10,000 spored oocytes in 0.5 ml inoculum in a single dose. The duration of the experiment was limited to 18 days. All 175 chicks (94 infected and 81 controls) were sacrificed by cervical dislocation between hours 1 and 432 following inoculation.

Immunohistochemical Techniques

Samples of the cecum, ileum, colon, rectum, and bursa of Fabricius were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned in 5-6 µm, and then mounted on glass slides. The sections were stained using the avidin-biotin-complex (ABC) technique (Shandon Laboratories, Pittsburgh, PA, USA). Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide in methanol for 20 min. After washing in PBS, 10% normal goat serum (Shandon Laboratories) was applied to sections to block non-specific binding of immunoglobulins. The sections were incubated with primary antibodies at a dilution of 1:400 in PBS (pH 7.4) for 60 min. As the primary antibody, monoclonal antibodies to each of the S100 subunits (α and β), a mouse anti-S100α (clone SH-A1, Sigma) and 2 mouse anti-S100β monoclonal antibodies (clones SH-B1 and SH-

B4, Sigma), or a rabbit anti-S100 (whole molecule) polyclonal antibody (Sigma). A Shandon Universal ABC Kit (Shandon Laboratories) was used to detect to binding of the primary antibody to the S100 protein. The sections were incubated with biotinilated anti-mouse/rabbit polyvalent secondary antibodies (Shandon) for 30 min. An avidin-peroxidase complex (Shandon Laboratories) was then applied for 30 min. For color reaction, 3-amino-9-ethylcarbozole (AEC) chromogen (Shandon Laboratories) was applied to the sections for 10-15 min (controlled by visual observation with a microscope). Sections were rinsed with distilled water, counterstained with Gill's hematoxylin for 1-2 min, and mounted with aqueous mounting medium (Shandon Laboratories). All steps were performed in a humidity chamber at room temperature. A positive and a negative control were used in each immunostaining to assess the specificity of the immunostaining method. Sections of chicken brains and hearts were used as positive controls for S100β - S100 whole and S100α, respectively. As negative controls, goat serum instead of primary antisera was applied to sections of samples, with the rest of the procedure being the same. The negative control sections were devoid of positive reacting parasite stages.

Results

When the sections were incubated with anti-S100 (whole molecule), anti-S100β, and anti-S100α primary antibodies, the second generation schizonts, gamonts, and oocytes in the ceca of the chicks infected with *E. tenella* were stained positive with all the 3 antibodies (Figures 1-4). While the schizonts that were positively stained with all the 3 antibodies were distributed throughout the lamina propria of the cecum, immunopositive macrogametes, microgametes, and oocytes were detected within the epithelial cells lining the deep portion of the crypts of Lieberkühn. However, the oocytes in the crypts' lumens were stained either positive or negative.

Immunopositive macrogametes, microgametes and oocytes showed a granular pattern of staining and were clearly outlined by a strong peripheral positive immunoreaction. In addition, the same stages of *E. tenella* were devoid of immunostaining.

A similar staining pattern was also observed in the endogenous stages of *E. tenella* located in the ileum, colon, and bursa of Fabricius of the infected chicks (Figures 5-8).

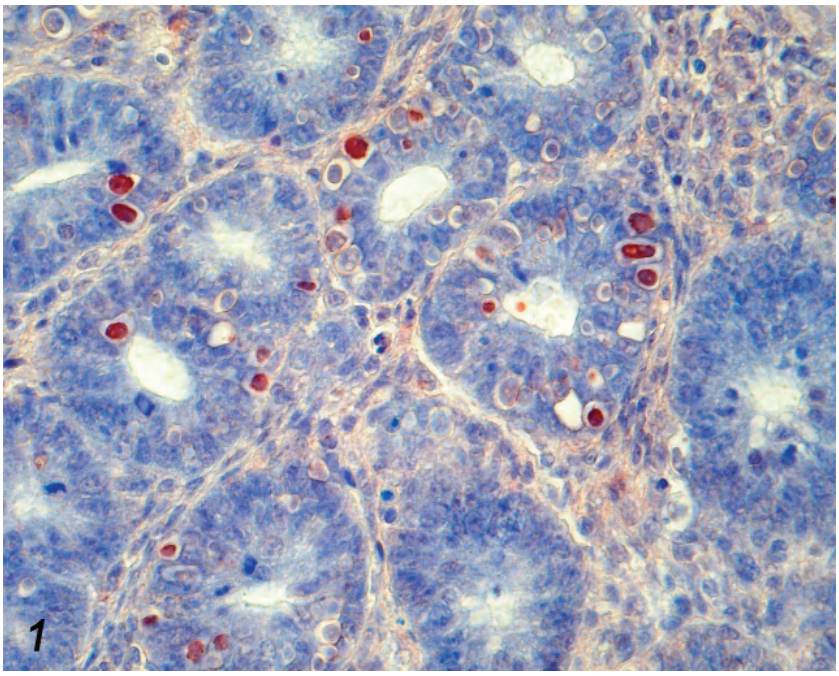

Figure 1. S100 whole molecule-positive merozoites in the cecal lamina propria from a chick killed 144 h PI. (Immunoperoxidase staining, AEC ×200).

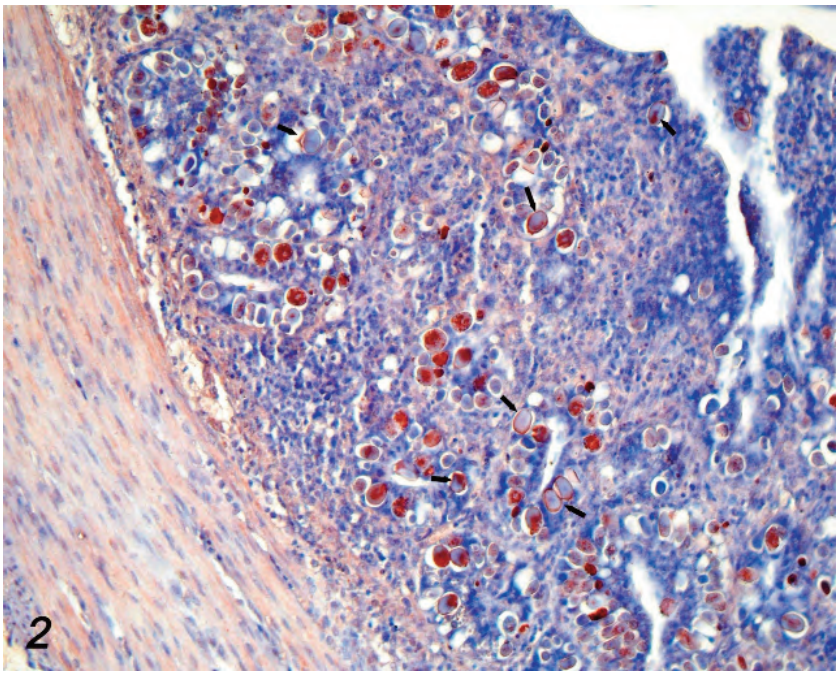

Figure 2. S100β-positive macrogametes and oocytes (arrows) in the cecal lamina propria from a chick killed 180 h PI. (Immunoperoxidase staining, AEC ×200).

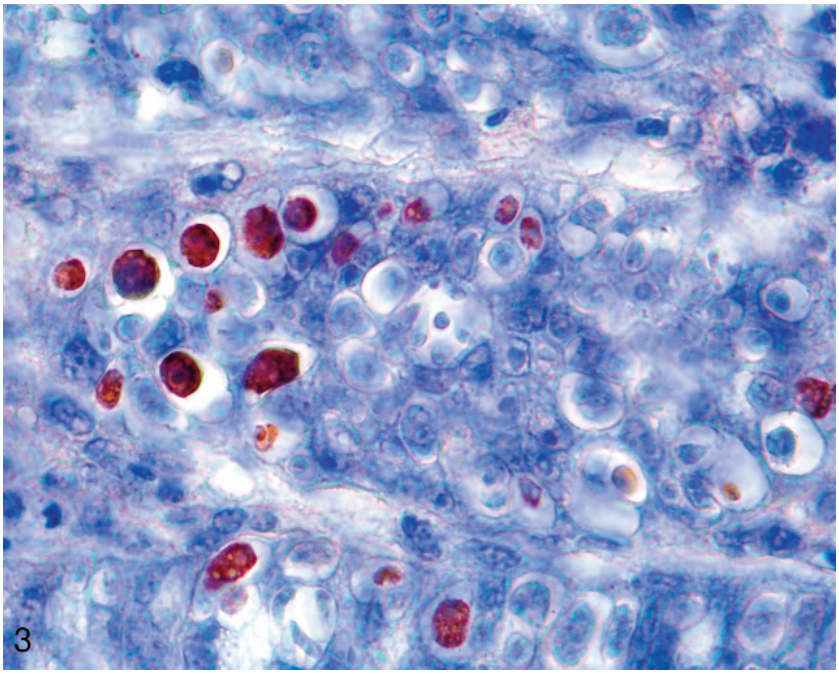

Figure 3. S100α-positive merozoites in the cecal lamina propria from a chick killed 144 h PI. (Immunoperoxidase staining, AEC ×1000).

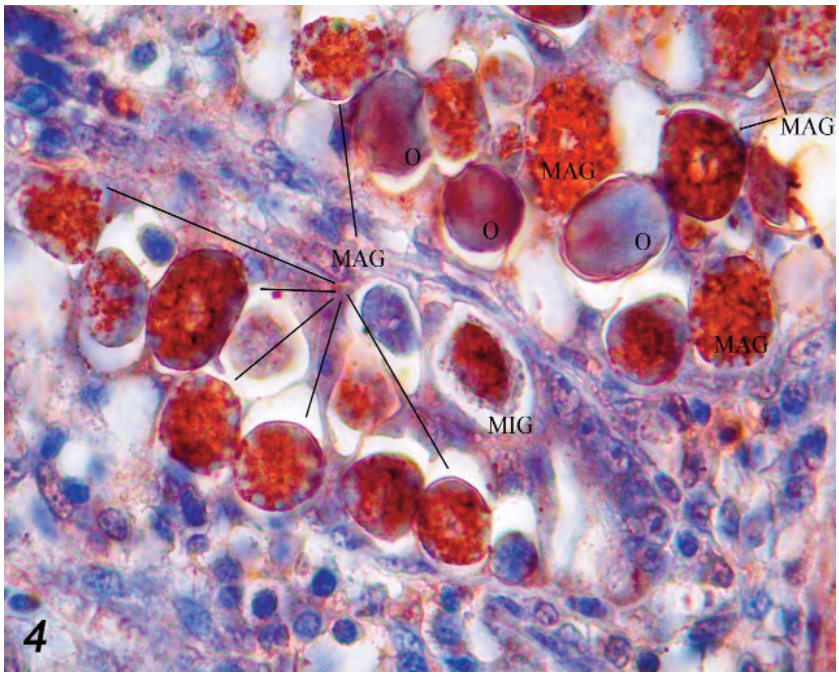

Figure 4. S100β-positive macrogametes (MAG), microgametes (MIC), and occytes (O) in the cecal lamina propria from a chick killed 180 h PI. (Immunoperoxidase staining, AEC ×1000).

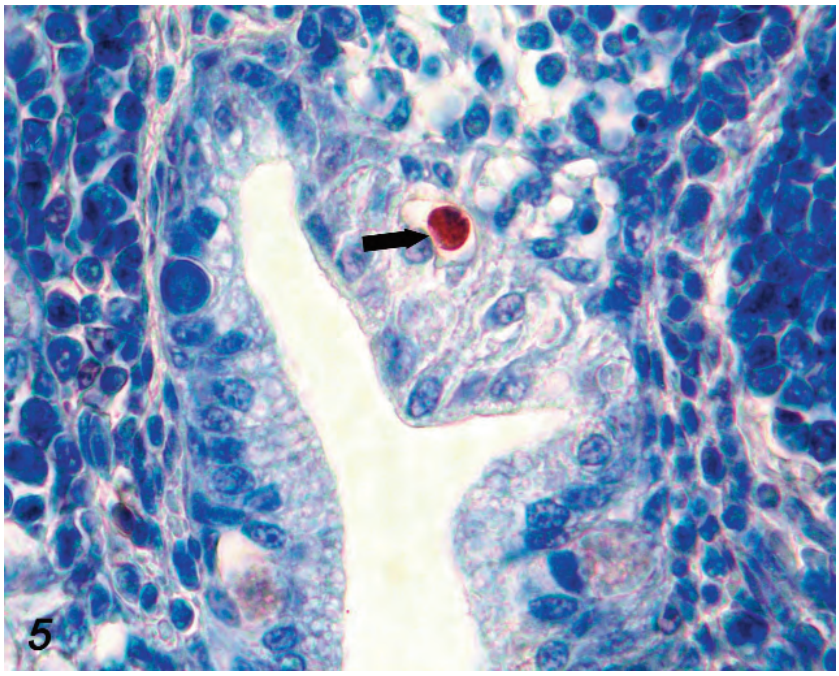

Figure 5. S100 whole molecule-positive (arrow) and negative meronts in the epithelium of the bursa of Fabricius from a chick killed 180 h PI. (Immunoperoxidase staining, AEC ×1000).

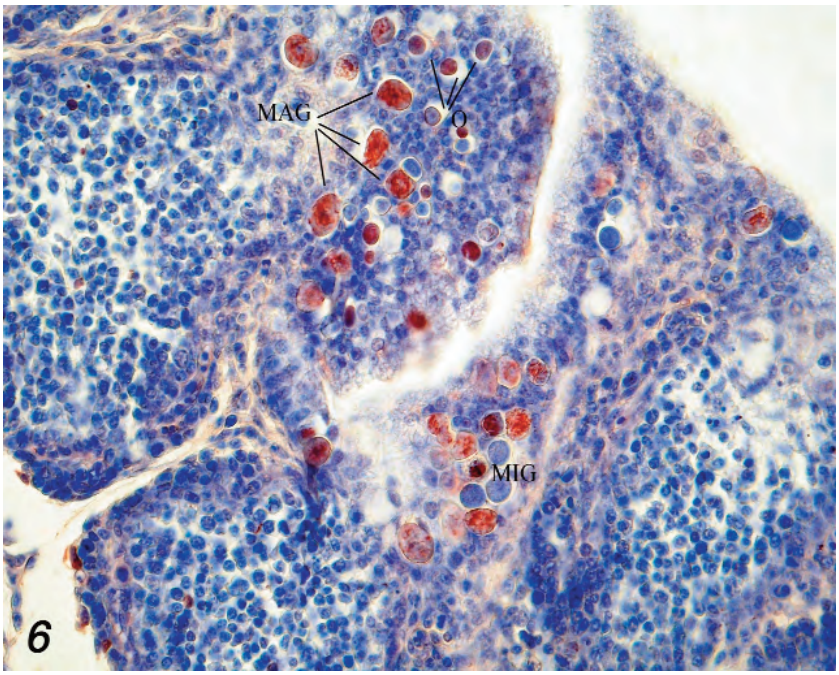

Figure 6. S100α-positive macrogametes (MAG), oocytes (O) and microgametes (MIG) in the epithelium of the bursa of Fabricius from a chick killed 180 h PI. (Immunoperoxidase staining, AEC ×1000).

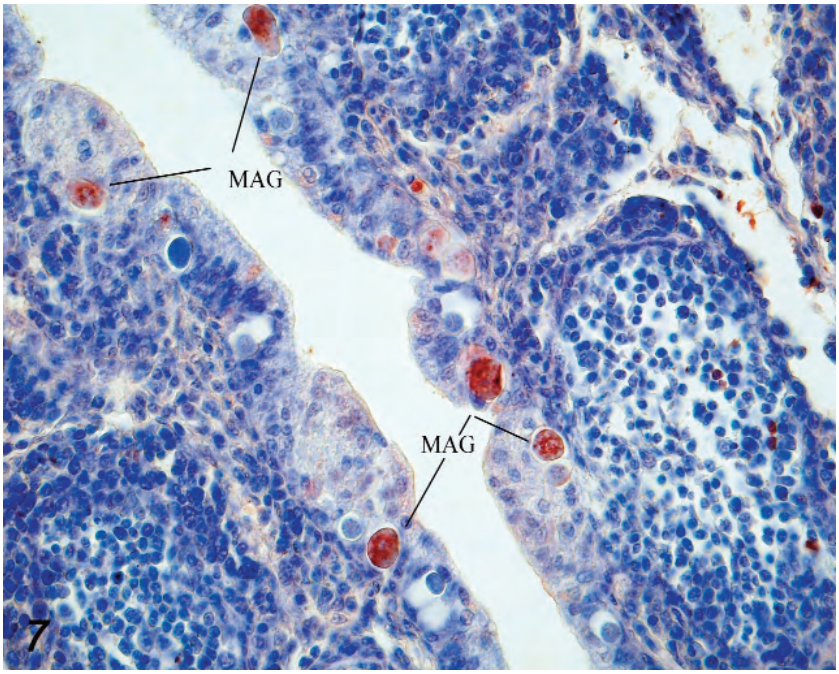

Figure 7. S100β-positive macrogametes in the epithelium of the bursa of Fabricius from a chick killed 180 h PI. (Immunoperoxidase staining, AEC ×400).

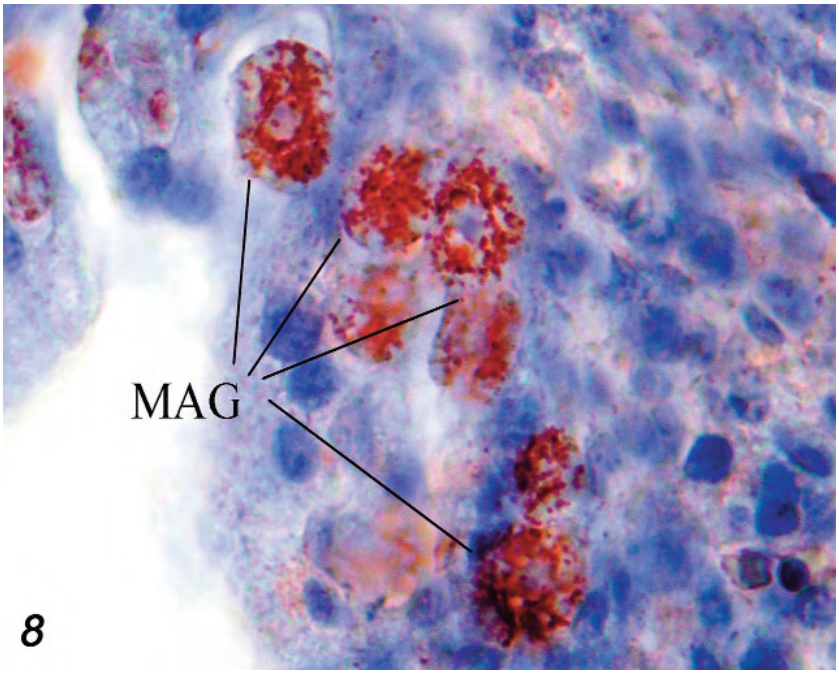

Figure 8. S100β-positive macrogametes in the epithelium of the bursa of Fabricius from a chick killed 180 h PI. (Immunoperoxidase staining, AEC ×1200).

Discussion

In the present study, the expressions and localizations of the β and α subunits of the S100 protein were investigated in all the endogenous phases of the intracellular protozoal parasite *E. tenella*. The immunoreactivity for the β and α subunits of the S100 protein was determined in the stages of *E. tenella* in the cecum where it is usually localized or develops and in organs where it is occasionally located such as the bursa of Fabricius, ileum, colon, and rectum.

In previous studies, a calmodulin-like domain bound to the catalytic domain of the calcium-dependent kinases was defined in some protozoa such as *Plasmodium falciparum* (17), *E. maxima* (18), and *E. tenella* (19). Furthermore, in subsequent studies, the presence of an S100-like protein was shown in some endogenous phases of *E. tenella* (14) and *E. brunetti* (15). This study is similar to recent studies on calcium-binding proteins in protozoa.

While del Cacho et al. (14) have defined an S100-like protein in the schizonts, macrogametes, and oocytes of *E. tenella* in the cecum, López-Bernad et al. (15) have demonstrated with immunohistochemical methods that this protein existed in the *E. brunetti* macrogametes located in the ileum and bursa of Fabricius epithelia. The results reported del Cacho et al. (14) revealed a positive immunoreaction for the S100 whole molecule and the β subunit in some developmental phases of *E. tenella*, while they displayed a negative reaction for the α subunit. Similarly, López-Bernard et al. (15) detected a positive immunoreaction for the S100 whole protein and the β subunit in ileal epithelia within macrogametes of *E. brunetti*. Moreover, they observed positive immunostaining for the α subunit as well in the macrogametes in the epithelium of the bursa of Fabricius. In our study, the localization of the S100 protein was investigated in the endogenous forms of *E. tenella* that penetrated into various other organs such as the ileum, colon, and bursa of Fabricius, in addition to the cecum, the primary area of development of the parasite. With the respect to the localization of S100 protein in *E. tenella*, the current results showed both similarities and differences when compared to the findings of the above-mentioned authors. In the present study, an immunoreaction was observed in the microgametes of the *E. tenella*, in addition to its schizonts, macrogametes, and oocytes. Furthermore, in our study, contrary to that reported by del Cacho et al. (14), all the endogenous

phases of the parasite displayed a reaction with not only the anti S100 whole molecule and anti S100β antibodies, but also with anti S100α antibody.

The subunits β and α of S100 are homologous molecules, and these proteins have various similar functions such as microtubule assembly-disassembly, protein phosphorylation, Ca^{2+} homeostasis, cell-cell communication, cell development, cell structure, energy metabolism, differentiation, contraction, apoptosis, immune response, and intracellular signal transduction (2,6,20,21). Although the exact roles of S100β and S100α subunits in *E. tenella* and *E. brunetti* are not known, these molecules probably function as calcium-binding molecules in these parasites (3,4). When it is taken into consideration that S100 proteins have various different functions as calcium-binding molecules, the following explanation can be made for the possible roles of this molecule in the developmental phases of *E. tenella*. S100A1 and S100B are important proteins taking part in microtubule assembly-disassembly, and can also regulate microtubule-mediated movement (3,4). As microtubules regulate the alterations in the cell morphology and polarity, the positive immunoreaction observed in the sporozoites and merozoites may be related to the active movement that these parasite stages develop before host cell invasion. Similarly, S100A1 and S100B subunits in macrogametes may be related to the movement of the wall-forming bodies in the cytoplasm to the macrogamete-limiting membrane to shape the oocyte wall. This movement can be related to an interaction with the microtubules that are important in the direct movement of the cytoplasmic organelles. Nevertheless, the positive immune reaction observed in the microgametes is related to the penetration of these forms of the parasite into the host cells including macrogametes.

Furthermore, a host intestinal immune response is known to be formed against parasites during *Eimeria* infections. Antibodies produced by the plasma cells, cytokines and lymphokines released by the T-lymphocytes and natural killer cells, mast cells, and macrophages are the factors playing roles in the intestinal immune response of the host (22). In our study, the negative immunoreaction observed in some stages of *E. tenella* was thought to be related to the loss of viability of some *E. tenella* forms as a result of the intestinal immune response.

The molecular mechanism of the infection caused by intracellular parasites in the host cells is not known well. Investigations on the invasion of the host cells by intracellular parasites have revealed the functions of some macromolecules during invasion (15). The gradually growing evidence shows that protein kinases (19), microtubules (11), and fibronectin (23) mediate attachment of the protozoan parasites to the host tissues during the invasion. Our results revealed that S100 proteins may be important macromolecules in the endogenous developmental stages of *E. tenella* in the host cells.

Consequently, knowledge of the roles of various molecules such as S100 proteins in intracellular protozoal parasitic infections will help us to understand the molecular mechanisms of parasitic development in host cells.

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