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REGULAR ARTICLE

The origin of IgG-containing cells in the bursa of Fabricius

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Abstract The bursa of Fabricius of the chicken is known as a primary lymphoid organ for B-cell development. Morphologically, the origin of IgG-containing cells in the bursa has not been clear until now, because abundant maternal IgG (MIgG) is transported to the chick embryo and distributed to the bursal tissue around hatching. Thus, it has been difficult to find out whether these cells themselves biosynthesize IgG or if they acquire MIgG via attachment to their surface. Our present study employing in situ hybridization clarified that IgG-containing cells in the medulla of bursal follicles did not biosynthesize IgG. To study the role of MIgG in the development of those IgG-containing cells, MIgG-free chicks were established from surgically bursectomized hen (SBx-hen). We found that, on the one hand, deprivation of MIgG from chicks completely inhibited the development of IgG-containing cells in the medulla after hatching. On the other hand, administration of MIgG to MIgG-free chicks recovered the emergence of those cells. In addition, we observed that those cells did not bear a B-cell marker and possessed dendrites with aggregated IgG. These results demonstrate that IgG-containing cells in the medulla are reticular cells that capture aggregated MIgG. Moreover, we show that the isolation of the bursa from environmental stimuli by bursal duct ligation (BDL) suppressed the development of IgG-containing cells after hatching. Thus, it is implied that environmental stimulations play a key role in MIgG aggregations and dendritic distributions of aggregated MIgG in the medulla after hatching.

Keywords The bursa of Fabricius · Maternal IgG · IgG-containing cells · Reticular cell · GALT

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Abbreviations

BDL	bursal duct ligation
CLSM	confocal laser scanning microscopy
FAE	follicle-associated epithelium
FDC	follicular dendritic cell
GALT	gut-associated lymphoid tissue
MIgG	maternal IgG
SBx-hen	surgically bursectomized hen

Introduction

In birds, the bursa of Fabricius is a primary lymphoid organ for B-cell proliferation and diversification (Glick et al. 1956; Masteller et al. 1995). The proliferation of B cells is

observed in the medulla of the bursa from day 14 of incubation (Masteller et al. 1995). Since a chick embryo is isolated from the external environment, the proliferation and somatic mutation of B cells in the medulla are regulated in an antigen-independent manner before hatching (Thorbecke et al. 1968; Kincade and Cooper 1971).

The emergence of IgG-containing cells in the bursa as a primary lymphoid organ gives rise to an idea that a developmental switch may sequentially occur in the bursa without antigenic stimulations (Kincade and Cooper 1971), due to the fact that IgG-containing cells are detected in the medulla around hatching (Thorbecke et al. 1968; Kincade and Cooper 1971; Ekino et al. 1995). However, IgG-containing cells are difficult to identify at the neonatal stage, because a dramatic increase in maternal IgG in the fetal circulation causes the diffuse distribution of MIgG over the bursal tissue in the last few days before hatching (Kowalczyk et al. 1985). In addition, bursal B cells preferentially bind MIgG with a Fcγ receptor (Viertboeck et al. 2007). Thus, accumulations of MIgG in the medulla cause difficulty in distinguishing between cytoplasmic and extracellular immunoglobulins. So to resolve this difficulty, we performed two experiments. Firstly, we studied the development of IgG-containing cells and IgG biosynthesizing cells (Cγ gene-expressing cells) in the bursa. In this experiment, we tried to prove our hypothesis that IgG-containing cells do not biosynthesize IgG in the medulla in situ (Ekino et al. 1995). Secondly, we established MIgG-free chicks from SBx-hens with agammaglobulinemia (Yasuda et al. 1998). MIgG-free chicks enabled us to study the role of MIgG in the development of IgG-containing cells in the medulla.

The bursa of Fabricius is also a gut-associated lymphoid tissue (GALT) in the form of a blind diverticulum connected to the cloaca (Schaffner et al. 1974; Sorvari et al. 1975) (Fig. 1a). The bursal lumen is occupied by vertical plicae containing about 10,000 follicles (Fig. 1c) (Olah and Glick 1978). The medulla develops in epithelium and it is isolated from systemic circulation by a basement membrane (Fig. 1b). After hatching, environmental material in the gut is conveyed into the bursal lumen by the tapping mechanism of the bursa (Schaffner et al. 1974; Sorvari et al. 1975; Ekino et al. 1985). Furthermore, it is taken up by FAE (follicle associated epithelium) and transported into the medulla (Fig. 1b, d). Since antigenic stimulations to the bursa from the cloaca influence B-cell proliferation and differentiation after hatching, B-cell development in the bursa is thought to be partly regulated by environmental antigens (Ekino et al. 1979b, 1980, 1985, 1993, 1995). Inactive particulate antigens such as Indian ink are found in the medulla after application to the cloaca (Schaffner et al. 1974; Sorvari et al. 1975; Sorvari and Sorvari 1977). On the other hand, active antigens such as bacteria and bovine serum albumin are not detected in the bursa (Schaffner et al. 1974; Sorvari and Sorvari 1977). It is thought that digestive enzymes in the medulla quickly degrade most environmental antigens derived from the cloaca (Grossi et al. 1977). In our previous study, the deprivation of environmental antigens from the bursa by BDL inhibited the emergence of IgG-containing cells in the medulla 1 week after hatching (Ekino et al. 1995). Thus, we further studied the effect of BDL on the development of IgG-containing cells in the medulla and the cortex.

Materials and methods

Chickens

Fertile eggs of partly inbred H.B15 strain (Kumamoto, Japan) and outbred White Leghorn chickens (Mie and Kumamoto, Japan) were used. Eggs were incubated and hatched in our own facilities. The Animal Care and Use Committee of the Kumamoto University Graduate School of Medical Sciences approved all procedures performed in this study.

Development of MIgG-free chicks

MIgG-free chicks were prepared from SBx-hens with agammaglobulinemia (Yasuda et al. 1998). SBx on day 18 of incubation eliminated IgG synthesis from hen. When SBx-hens were housed in an isolated clean room and antibiotics were administered to them with food and water, they could survive until sexual maturity. SBx-hens in which serum IgG was less than 0.05 mg/ml were selected as IgG-free hens 11 weeks after hatching. Concentrations of yolk IgG in six fertilized eggs of normal hens were 27.1±8.0 mg/ml. On the other hand, those of six IgG-free hens were 0.002± 0.002 mg/ml. Finally, we succeeded in obtaining MIgG-free chicks from SBx-hens. Concentrations of serum IgG in seven MIgG-free chicks were 0.04±0.02 mg/ml 5 days after hatching (Yasuda et al. 1998). Cryostat sections of bursal tissue in normal and MIgG-free chicks were studied 1 week after hatching.

Purification of maternal IgG from yolk

The yolk was separated from the egg white and yolk membrane and it was mixed with 8 volumes of distilled water. An aqueous suspension of 5 % hydroxypropylmethylcellulose phthalate in 80 % ethyl alcohol was added to the diluted egg yolk gradually, while mixing evenly; the mixture was then incubated at 10°C for 18 hours (Yokoyama et al. 1993). The supernatant was collected and passed through a 0.45-μm membrane filter.

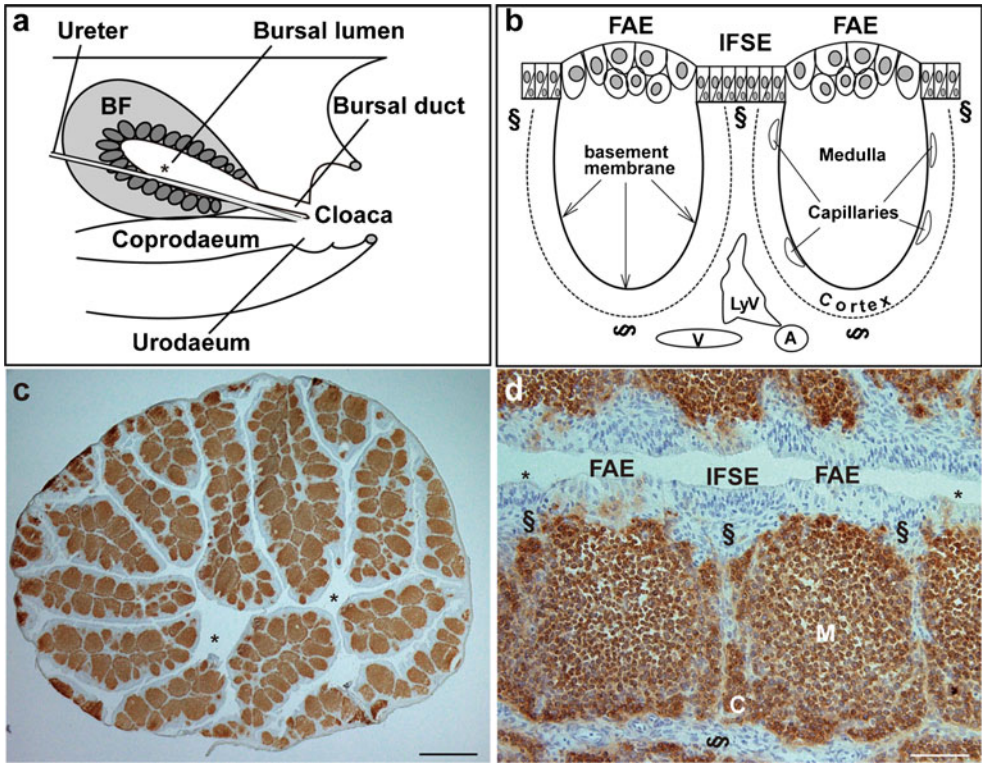

Fig. 1 Anatomical and histological schemata of the bursa of Fabricius. **a** Anatomical explanation of the bursa. **b** Histological explanation of bursal follicles. **c,d** Frozen sections of the bursa in a 3-day-old chicken were stained with anti-Bu1b mAb, which reacts with the chicken B cell surface antigen. B cells are recognized as brownish cells. **a** The bursa is a lymphoid organ in the form of a blind sac connected by a narrow bursal duct to the dorsal part of the cloaca. **b,d** The individual follicle is composed of the medulla (*M*) and the cortex (*C*). There are two types of epithelium: *FAE* (follicle associated epithelium) and *IFSE* (interfollicular surface epithelium) (Naukkarinen 1978). The FAE is polygonal and larger than the pseudostratified columnar IFSE. The basement membrane forms the boundary between the cortex and the medulla. Capillaries form a network along the basement membrane in the cortex and do not penetrate into the medulla. The lymph vessels (*LyV*) are located in the connective tissue of lamina propria (*§*) (Ekino et al. 1979a). **c** The bursal lumen (*) is occupied by more than ten thick, vertical plicae (Olah and Glick 1978). **c** *Scale bar*=500 μm. **d** *Scale bar*=50 μm

Intravenous injection of FITC-labeled maternal IgG

Purified yolk IgG was conjugated with FITC (Dojindo, Kumamoto, Japan) according to Goding (1976). FITC-MIgG (2 mg/0.1 ml) was injected into a chorioallantoic vein of a MIgG-free chick embryo on day 18 of incubation. Cryostat sections of bursal tissue were studied 5 days after hatching and on day 19 of incubation by confocal laser scanning microscopy (CLSM) (Leica and Olympus).

Isolation of the bursa of Fabricius from the gut by bursal duct ligation (BDL)

BDL was performed without damaging the arterial supply and lymphatic drainage on day 18 of incubation as described previously (Ekino at al. 1979a, 1980, 1993).

Antibodies

mAbs specific for chicken Cμ, Cγ and Bu1 Ag were used. HIS C12 (anti-Cμ) was generated and characterized in the University of Groningen and the Central Veterinary Institute-DLO (Jeurissen et al. 1988). M-1 (anti-Cμ) was purchased from Sera-lab (Sussex, England) (Chen et al. 1982). CVI-ChIgG-47.3 (anti-Cγ) was generated and characterized at the Central Veterinary Institute (Koch and Jongenelen 1989). The specificity of mAbs for the Cμ and Cγ heavy chain of chicken Ig was demonstrated in our previous report (Ekino et al. 1995). L22 (anti-Bu1a) and 11 G2 (anti-Bu1b) were generated in the Basel Institute for Immunology (Pink and Rijinbeek 1983) and Turku University respectively (Veromaa et al. 1988).

Immunoperoxidase staining of tissue sections

For cryostat sections, small fragments of the bursa were snap-frozen with liquid nitrogen at −196 °C. These cryostat sections (10 μm) were air-dried for at least 30 min, fixed in pure acetone for 10 min at room temperature and air-dried again for 15 min. Sections were treated with 100 % cold methanol containing 0.3 % H_2O_2 for 10 min to remove endogenous peroxidase activity (Ekino et al. 1995). After

rinsing in PBS, sections were overlaid with mAb and incubated for 1 h at room temperature. Subsequently, the sections were rinsed in PBS three times and incubated for 30 min with peroxidase-conjugated polyclonal horse anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA, USA) or peroxidase-conjugated polyclonal goat anti-mouse IgG (Nichirei Bioscience Inc., Tokyo, Japan). These second-stage antibodies conjugated to peroxidase did not cause any unspecific staining (Fig. 2). After rinsing in PBS, peroxidase activity was demonstrated by applying 3,3'-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan) at a concentration of 0.5 mg/ml in 0.05 M Tris–HCl buffer (pH 7.6) containing 0.01 % H_2O_2. The enzyme reaction generated a brownish, dense, electron-opaque product. The reaction product was intensified by immersing the sections for 5 min in 0.5 % $CuSO_4$ in 0.9 % NaCl. Hematoxylin staining according to Mayer was used as a counterstain. The number of samples examined on day 18 and 21 of incubation, 3 days, 1 week, 2 weeks and 3 weeks after hatching was four to nine. Some sections were washed for 10–30 minutes with clod PBS prior to fixation.

Immunofluorescence double staining of tissue sections

Cryostat sections (10 μm) were air-dried for at least 30 min, fixed in pure acetone for 10 min at room temperature and air-dried again for 15 min. These cryostat sections were exposed with unlabeled mAb for 1 h. These were incubated with TRITC-conjugated goat anti-mouse IgG (Fab specific) (Sigma) for 30 min. After rinsing in PBS, unbound sites of goat anti-mouse IgG were blocked with 5 % normal mouse serum for 30 min. After rinsing in PBS, second-step labeling was done with biotinylated mAb followed by ExtraAvidin®-FITC conjugate (Sigma). After washing in PBS, the sections were mounted and coverslipped with Vectashield® (Vector Laboratories Inc., Burlingame, CA, USA). The cryostat sections were studied by CLSM.

Whole-mount in situ hybridization

The primers were designed, with addition of either BamHI or EcoRI sites, from the nucleotide sequence of the EMBL/GenBank/DDBJ data bases: 5′ of Cm exon 3; CM3, AGTG GATCCGTCGTGCAGCAGGACAT: 3′ of Cm exon 4; CM2, ATCGAATTCACCCGAAGCCCTATCCA: 5′ of Cg exon 3; CG3, AGTGGATCCGACGGCGCTCAGAGCTG: 3′ of Cg exon 4; CG4, GCGGTGGTGACGAATTCGGTGGCGGG. The bCM3 and bCG2 were constructed by inserting the 0.7 kb PCR fragment of the chicken Cμ gene and the 0.5 kb PCR fragment of the chicken Cγ gene, respectively. Digoxigenin (DIG) -UTP-labeled RNA probes for hybridization were prepared using the plasmids bCM3 and bCG2. BamHI-linealized bCM3 and bCG2 were transcribed with T3 RNA polymerase for making antisense probes and EcoRI-linealized bCM3 and bCG2 with T7 RNA polymerase for making sense probes. Whole-mount in situ hybridization was performed in the manner described previously (Shimamura et al. 1994). The number of samples examined on day 18 of incubation, 3 days, 2 weeks, and 3 weeks after hatching was two to five. Each sample was divided into two and studied for the distribution of Cμ gene-expressing cells and that of Cγ gene-expressing cells respectively.

Results

Development of immunoglobulin containing cells and immunoglobulin biosynthesizing cells in the bursa of Fabricius

Immunoglobulin-containing cells were detected by immunoperoxidase staining. However, it is not clear whether immunoglobulins detected by immunoperoxidase staining are

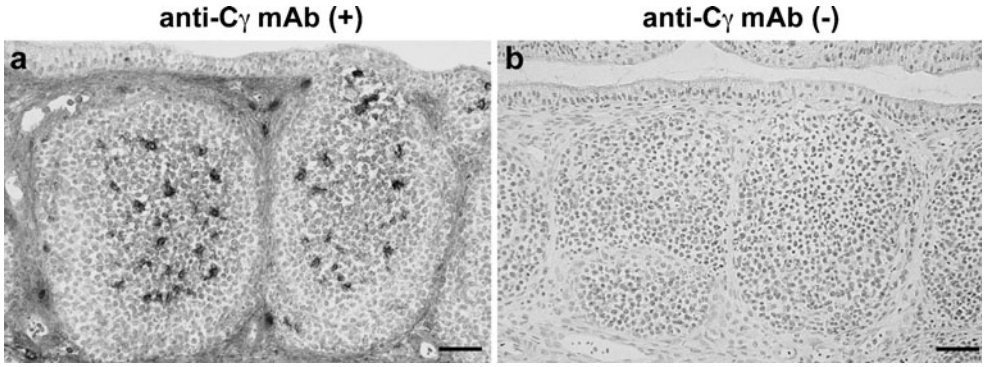

Fig. 2 A control staining of bursal tissue with the second-stage antibody conjugated to peroxidase. The bursa of a 1-week-old chicken was stained with anti-Cγ mouse mAb and peroxidase-conjugated polyclonal goat anti-mouse IgG (**a**). The serial section was stained with peroxidase-conjugated polyclonal goat anti-mouse IgG (**b**). The second-stage antibody conjugated to peroxidase did not cause any unspecific staining. *Scale bars*=20 μm

biosynthesized in situ or captured. Then, in situ hybridization was employed to determine whether immunoglobulin-containing cells biosynthesize immunoglobulin in situ or not.

Individual Cμ gene-expressing cells were clearly distinguished (Fig. 3b, d, f) and the cortex was easily detected (Fig. 3d, f). On the other hand, it was difficult to discriminate between individual IgM-containing cells and the cortex, due to extracellular IgM after hatching (Fig. 3c, e). On day 18 of incubation, IgM-containing cells were detected in the medulla but extracellular IgM was not found in the connective tissue of the lamina propria because of the absence of serum IgM (Hamal et al. 2006) (Fig. 3a). Thus, the distribution of IgM-containing cells coincided with that of Cμ gene-expressing cells in the bursa at any age examined. When we used a sense probe instead of an antisense probe, no staining was observed (data not shown).

On day 18 of incubation, IgG spread over the extracellular space of the lamina propria and the medulla but did not form aggregates (Fig. 4a). Neither IgG-containing cells nor Cγ gene-expressing cells were detected at all in the bursal tissue on day 18 of incubation (Fig. 4a, b). Meanwhile, IgG-containing cells were found in the medulla of the bursa 3 days and 2 weeks after hatching (Fig. 4c,e) although some of them were also seen in the lamina propria and the cortex of the bursa 2 weeks after hatching (Fig. 4e arrows). Those IgG-containing cells were reticularly distributed in the central area of the medulla and the number of them increased with age. On the other hand, Cγ gene-expressing cells were not detected at all in the medulla 3 days and 2 weeks after hatching (Fig. 4d, f). The difference between the distribution of IgG-containing cells and that of Cγ gene-expressing cells became more evident in the bursa of 3-week-old chickens (Fig. 5a, b). In an immunoperoxidase tissue section, the reticular distribution of IgG-containing cells were observed in the medulla and some IgG-containing cells were also distributed in the lamina propria and the cortex (Fig. 5a). Contrastingly again, Cγ gene-expressing cells were not detected at all in the medulla (Fig. 5b). Cγ gene-expressing cells were observed in the lamina propria and the cortex. Since these cells in the lamina propria show the cytoplasmic expression of Cγ gene in quantity (Fig. 5b inset), they are presumed to be IgG-producing plasma cells.

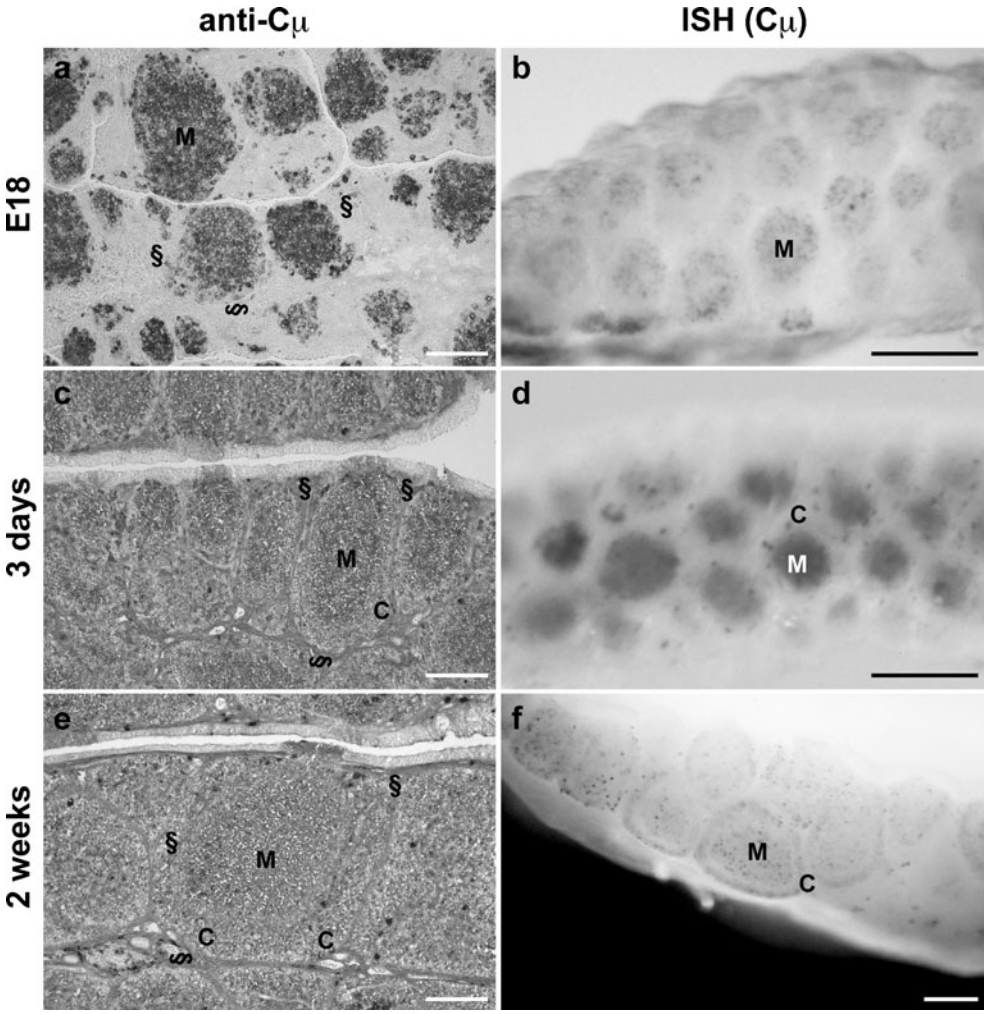

Fig. 3 Development of IgM-containing cells (**a,c,e**) and Cμ gene-expressing cells (**b,d,f**) in the bursa. IgM-containing cells in the bursa on day 18 of incubation (E18) (**a**), 3 days after hatching (**c**) and 2 weeks after hatching (**e**). Cμ gene-expressing cells in the bursa at day 18 of incubation (**b**), 3 days after hatching (**d**) and 2 weeks after hatching (**f**). **a** Before hatching, IgM-positive cells formed the medulla (*M*). Extracellular IgM was not observed in the connective tissue of the lamina propria. **c,e** After hatching, IgM-containing cells formed the medulla (*M*) and the cortex (*C*) and extracellular IgM was distributed in the lamina propria (*§*) and follicles. **b** Before hatching, Cμ gene-expressing cells formed the medulla (*M*) but not the cortex. **d,f** After hatching, Cμ gene-expressing cells were detected in the medulla (*M*) and the cortex (*C*). *Scale bars*=100 μm

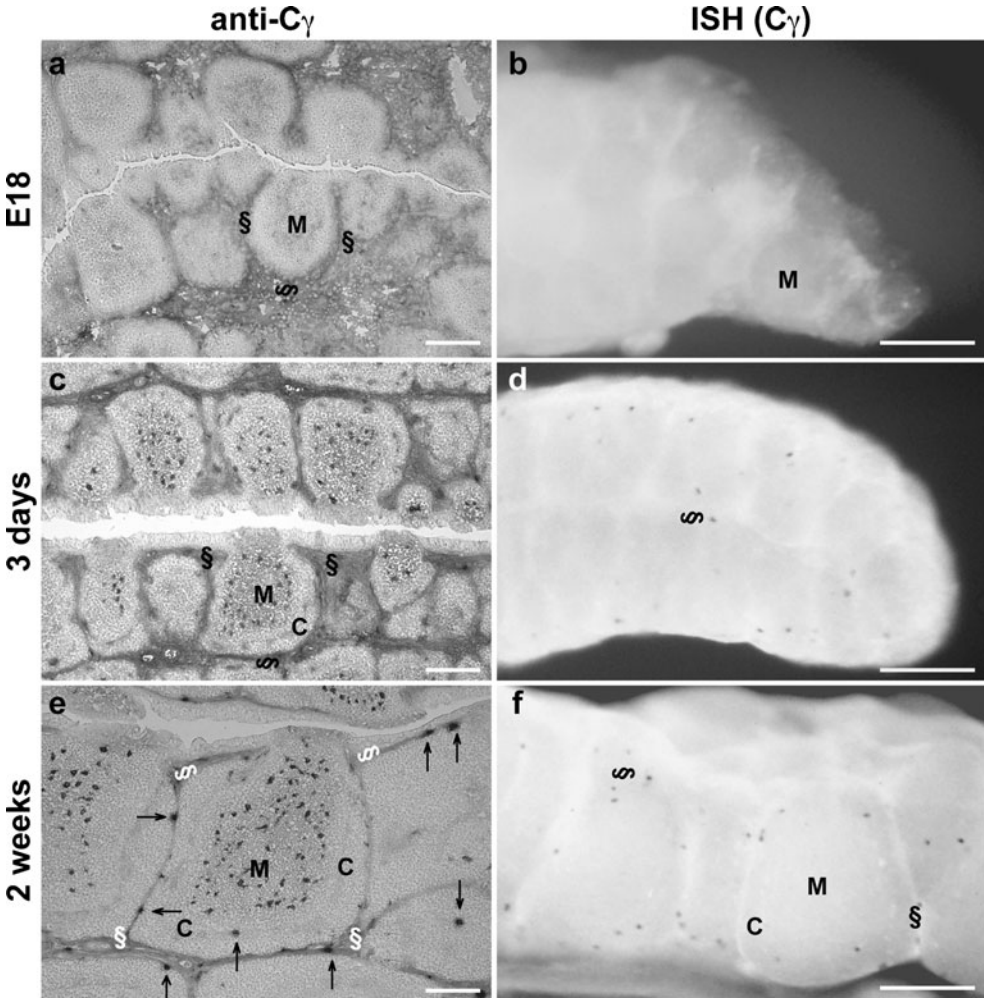

Fig. 4 Development of IgG-containing cells (**a,c,e**) and Cγ gene-expressing cells (**b,d,f**) in the bursa. IgG-containing cells in the bursa on day 18 of incubation (*E18*) (**a**), 3 days after hatching (**c**) and 2 weeks after hatching (**e**). Cγ gene-expressing cells in the bursa on day 18 of incubation (**b**), 3 days after hatching (**d**) and 2 weeks after hatching (**f**). **a** Before hatching, serum IgG derived from yolk was distributed over bursal follicles and the lamina propria (*§*). IgG-containing cells were not detected in the medulla (*M*). **c, e** After hatching, most of IgG-containing cells were detected in the medulla (*M*) and a few IgG-containing cells were seen in the cortex (*C*) and the lamina propria (*§*) (*arrows*). **b** Before hatching, no Cγ gene-expressing cells were detected in the bursa at all. **d, f** After hatching, Cγ gene-expressing cells were detected in the cortex (*C*) or the lamina propria (*§*) but not in the medulla. *Scale bars*= 100 μm

The localization of Cγ gene-expressing cells correlated with that of IgG-containing cells in the lamina propria and the cortex. These results reveal that IgG-containing cells in the medulla do not biosynthesize IgG at any age examined. Consequently, it is suggested that IgG-containing cells are induced by the attachment of IgG to bursal cells in the medulla. When we used a sense probe instead of an antisense probe, no staining was observed (data not shown).

Role of MIgG in the development of IgG-containing cells in the bursa

Mostly, IgG in neonatal chickens is derived from yolk (Kowalczyk et al. 1985). On the hypothesis that IgG-containing cells may be generated by attachments of MIgG to medullary bursal cells after hatching (Ekino et al. 1995), we developed MIgG-free chicks (Yasuda et. al. 1998) and examined the effects of deprivation of MIgG on the development of IgG-containing cells in the bursa.

In 1-week normal chickens, many IgG-containing cells were observed in the medulla of all bursal follicles and extracellular IgG was seen in the lamina propria (Fig. 6a, c, e). In contrast, in 1-week MIgG-free chicks, deprivation of MIgG resulted in a complete suppression of the emergence of IgG-containing cells in the medulla (Fig. 6b, d, f) and in addition, the lamina propria in MIgG-free chicks was not stained by anti-Cγ mAb. Absence of extracellular IgG in the lamina propria reconfirmed the lack of serum IgG in MIgG-free chicks. These results clarify that IgG-containing cells are induced by the attachment of MIgG after hatching. A few IgG-containing cells were observed in the lamina propria of both normal and MIgG-free chicks (Fig. 6c, d, e, f, arrows).

Administration of FITC-MIgG into a MIgG-free chick embryo via a chorioallantoic vein induced the recovery of IgG-containing cells after hatching (Fig. 7a). Subsequent to hatching, FITC-MIgG formed large dendritic aggregates in the medulla and small aggregates in FAE (Fig. 7a). On the other hand, before hatching, FITC-MIgG was diffusely distributed to the bursal follicles and the lamina propria but did not form any aggregates and dendritic distribution (Fig. 7b). These results reconfirm that IgG-containing cells in the

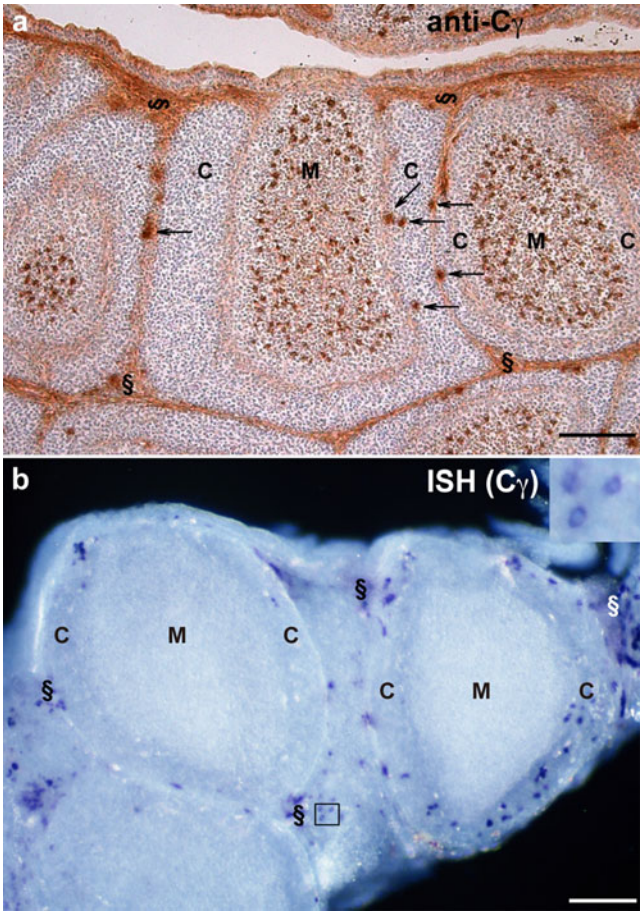

Fig. 5 Differences between the distribution of IgG-containing cells (**a**) and that of Cγ gene-expressing cells (**b**) in the bursa 3 weeks after hatching. **a** IgG-containing cells were observed in the medulla (*M*). Some IgG-containing cells were detected in the cortex (*C*) and in the interfollicular connective tissue (*arrows*). Extracellular IgG was distributed in the subepithelial connective tissue and the interfollicular connective tissue of the lamina propria (*§*). **b** Cγ gene-expressing cells were detected throughout the cortex (*C*) and in the connective tissue of the lamina propria but not at all in the medulla (*M*). Inset. The high magnification of the area indicated by the *rectangle* shows the expression of Cγ gene in the cytoplasm but not the nucleus. *Scale bars*= 100 μm

medulla are generated by the attachment of MIgG after hatching and that environmental stimuli from the gut after hatching play a key role in the aggregation of MIgG and the formation of IgG-containing cells in the medulla.

Morphological characteristics of IgG-containing cells in the medulla

As it is not clear whether IgG-containing cells are B cells or reticular cells, IgG-containing cells in the medulla were studied by two-color CLSM analysis using anti-Bu1 and anti-Cγ mAbs (Fig. 8). Bu1 and IgG were visualized with red and green respectively. Since CLSM has the ability of providing images of about 1-μm-thick optical sections, a cell membrane is recognized as a thin circle. B cells in the bursal follicle were recognized as red circles (Fig. 8a, b). IgG-containing cells showed a reticular distribution in the center of the medulla (Fig. 8c, d). Co-localization of IgG (green) with B-cell surface Ag (red) was studied by CLSM. IgG-containing cells in the medulla showed green with dendritic appearances but did not show yellow indicating co-localization of IgG and the B-cell surface Ag (Fig. 8e, f). Therefore, they are not B cells. Their diameter was about 20 μm (Fig. 8d, f). These results elucidate that IgG-containing cells in the medulla are reticular cells with dendritic appearances. As IgG-containing cells in the lamina propria were also Bu1-negative (Fig. 8c,e arrows) and secreted IgG around them, IgG-containing cells in the lamina propria or the cortex are IgG-producing plasma cells.

In immunoperoxidase staining, accumulated reaction products generated strong backscattered light signals. These signals were visualized by CLSM. In a transmitted light image, the accumulated reaction products were seen as black shadows (Fig. 9a). In a confocal backscattered light image, reaction products in IgG-containing cells were seen as bright (yellow) clusters (Fig. 9b). However, extracellular IgG produced insignificant signals. Then, it was indicated that IgG in IgG-containing cells forms aggregates (Fig. 9b).

Role of environmental antigens in the development of IgG-containing cells in the medulla

Our previous study suggests that environmental antigens play a key role in the emergence of IgG-containing cells in the medulla of bursal follicles (Ekino et al. 1995). So to study the role of environmental antigens in the development of IgG-containing cells in the medulla, we examined effects of BDL on the development of IgG-containing cells in the bursa. IgG-containing cells were always observed in the medulla of all bursal follicles 3 days, 1 week and 3 weeks after hatching (Fig. 10a, c, e). Deprivation of environmental antigens from the bursa by BDL completely suppressed the development of IgG-containing cells in the medulla, although serum IgG was diffusely distributed in the lamina propria and the medulla (Fig. 10b, d, f). IgG-producing plasma cells (arrows) were localized in the lamina propria and the cortex in both normal (Fig. 10a, c, e) and BDL-chickens (Fig. 10d, f). These results indicate that environmental antigens are a prerequisite for the development of IgG-containing cells in the medulla.

Medullary localization of MIgG in the bursa of Fabricius

MIgG is transported from the yolk to the chick embryo and reaches its highest levels in the serum of the chick embryo around hatching. As a result, plenty of MIgG have shown

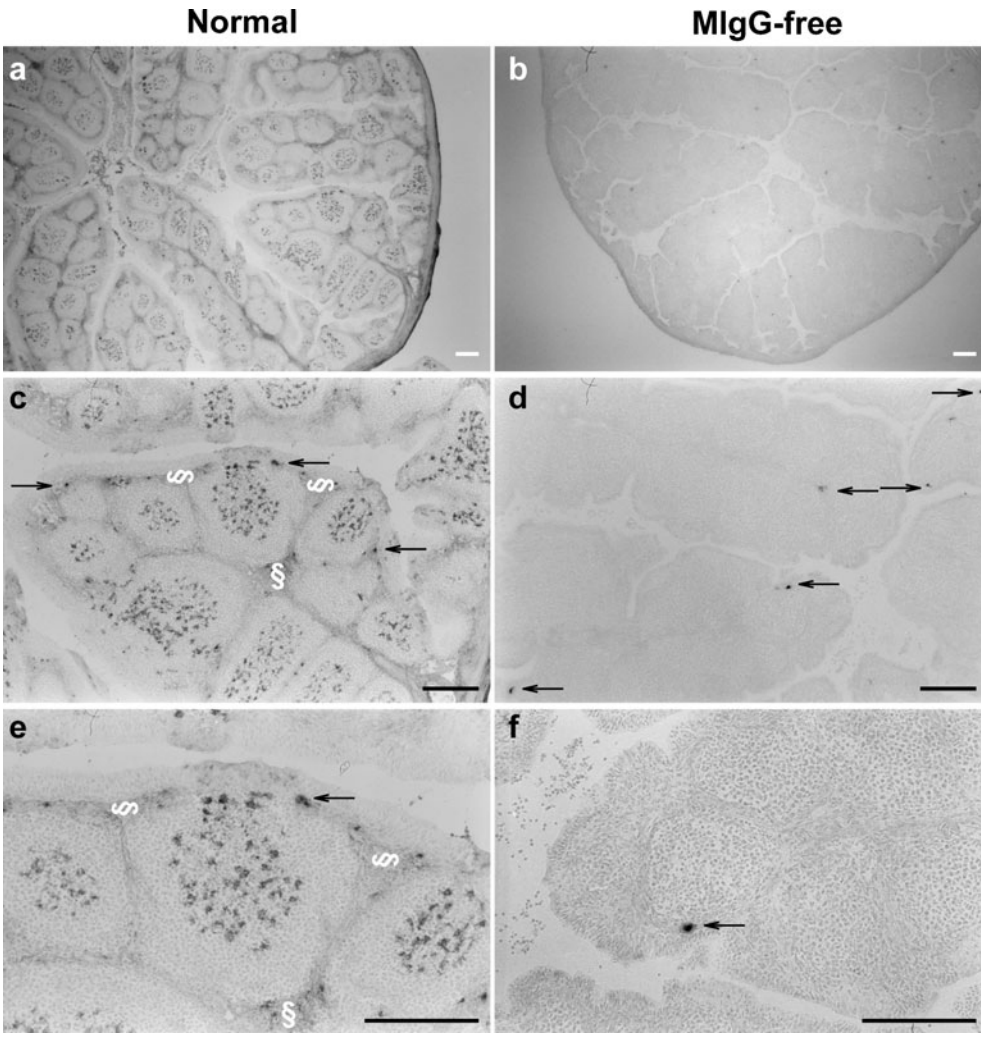

Fig. 6 Role of MIgG in the development of IgG-containing cells in the bursa. **a,c,e** The distribution of IgG-containing cells in the bursa of a normal chicken 1 week after hatching. **b,d,f** The distribution of IgG-containing cells in the bursa of a MIgG-free chick 1 week after hatching. **a** Low-power field of the cross section of the bursa in a normal chicken. IgG-containing cells were detected in all bursal follicles. **b** Low-power field of the cross section of the bursa in a MIgG-free chick. IgG-containing cells were not detected in any bursal follicles at all. Extracellular IgG was also not seen in any areas of bursal tissue. **c,e** IgG-containing cells were detected in the medulla. Also, serum IgG was distributed over the lamina propria (*§*). IgG-producing plasma cells were detected in the lamina propria (*§*) (*arrows*). **d,f** Deprivation of MIgG from the chick caused the complete suppression of the development of IgG-containing cells in the medulla 1 week after hatching. A few IgG-producing plasma cells (*arrows*) were detected in the lamina propria of the bursa in MIgG-free chicks. *Scale bars*=100 μm

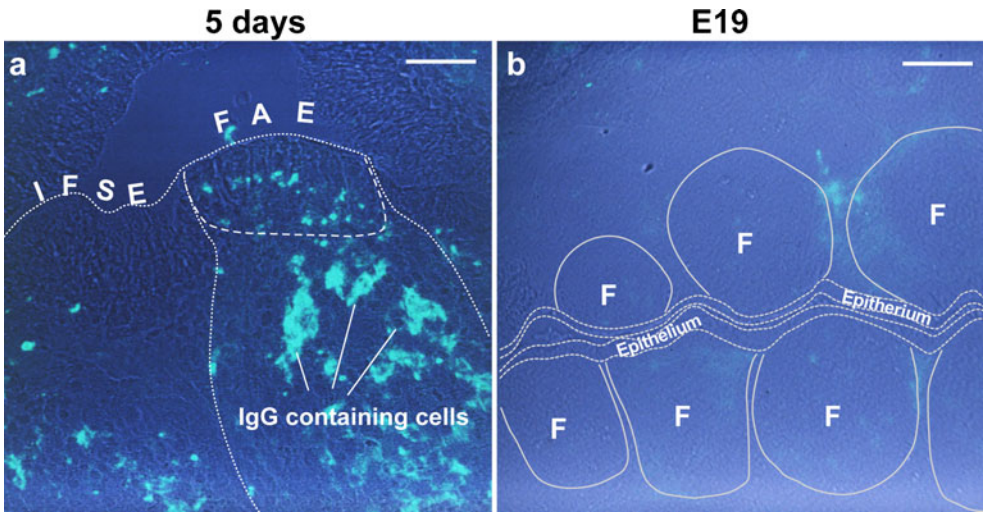

Fig. 7 Effect of administration of FITC-labeled IgG into a MIgG-free chick embryo on the emergence of IgG-containing cells in the bursa after hatching (**a**) and before hatching (**b**). **a** Distribution of FITC-MIgG in the bursal follicles 5 days after hatching (8 days after injection of FITC-MIgG into a chorioallantoic vein of a MIgG-free chick embryo on day 18 of incubation). Aggregates of FITC-MIgG formed IgG-containing cells with dendritic appearances in the medulla. FITC-MIgG was also detected as aggregates in FAE but not in IFSE. **b** Distribution of FITC-MIgG in the bursa on day 19 of incubation (*E19*) (1 day after injection of FITC-MIgG into a chorioallantoic vein of a MIgG-free chick embryo on day 18 of incubation). IgG-containing cells with aggregated FITC-MIgG were not seen in the bursal tissue before hatching. **a** *Scale bar*=10 μm. **b** *Scale bar*=100 μm

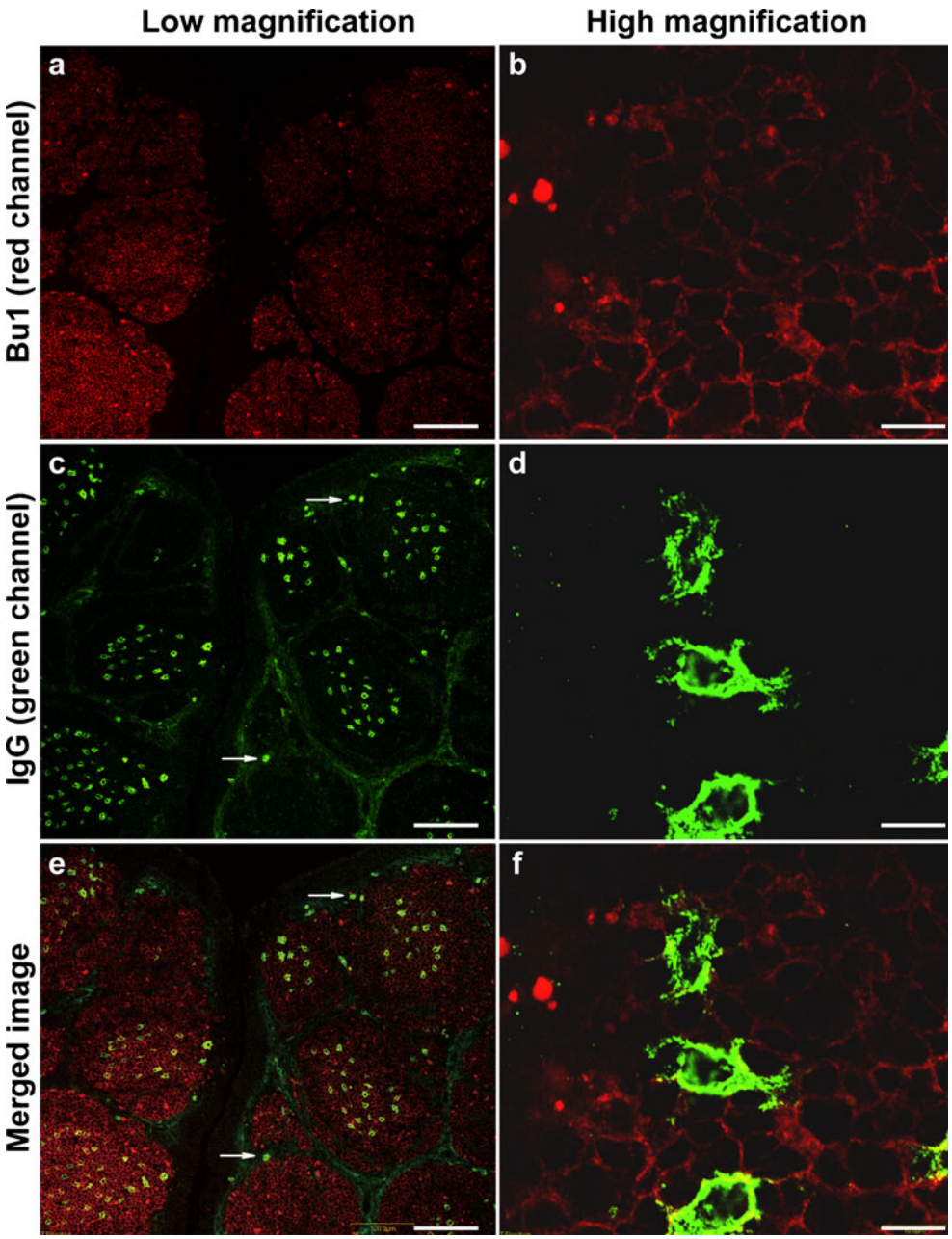

Fig. 8 CLSM images of IgG and/or Bu1 positive cells in the bursal follicle of a 1-week old chicken. **a,c,e** A CLSM image at low magnification. **b,d,f** A CLSM image at high magnification. **a,b** Bu1 was visualized with red. **c,d** IgG was visualized with green. **e,f** The red and green fluorescence images were recorded separately and merged. Co-localization is in yellow. IgG-containing cells in the medulla were green but not yellow (**f**). IgG-containing cells were distributed in the central area of the medulla. Bright IgG-positive cells in the lamina propria were Bu1-negative (*arrows*) (**c,e**). IgG-containing cells in the medulla showed large dendritic appearances and IgG appeared to be as accumulations of aggregates and distributed in the cytoplasm and on reticular branches (**d,f**). **a,c,e** *Scale bars*=100 μm. **b,d,f** *Scale bars*=10 μm

"colloid-like" accumulations in the medulla before hatching (Thorbecke et al. 1968; Kincade and Cooper 1971) (Fig.11a, c). The dense accumulations of MIgG disturb the distinction of IgG-containing cells in the medulla before hatching. Washing cryostat sections with cold PBS prior to fixation removed IgG from the follicles and the lamina propria (Fig. 11b, d, f). Thus, washing with cold PBS made it clear that IgG-containing cells did not emerge before hatching (Fig. 11b, d). The morphology of IgG-containing cells after hatching was preserved even after washing (Fig. 11f). These results demonstrate that, before hatching, MIgG is already distributed in the medulla of the bursal follicles and does not form aggregations.

Discussion

The distributions of IgM biosynthesizing cells in the bursa were almost identical to those of IgM-containing cells at all ages tested (Fig. 3). These histological results support a general knowledge that IgM-containing cells biosynthesize IgM in the bursa (Thorbecke et al. 1968; Kincade and Cooper 1971). Extracellular IgM was not detected in the lamina propria of the bursa before hatching because IgM was not detected in the serum before hatching (Hamal et al. 2006) (Fig. 3a). Thus, it is postulated that bursal B cells biosynthesize IgM and that they express as membrane IgM but do not secrete before hatching. Contrastingly, the

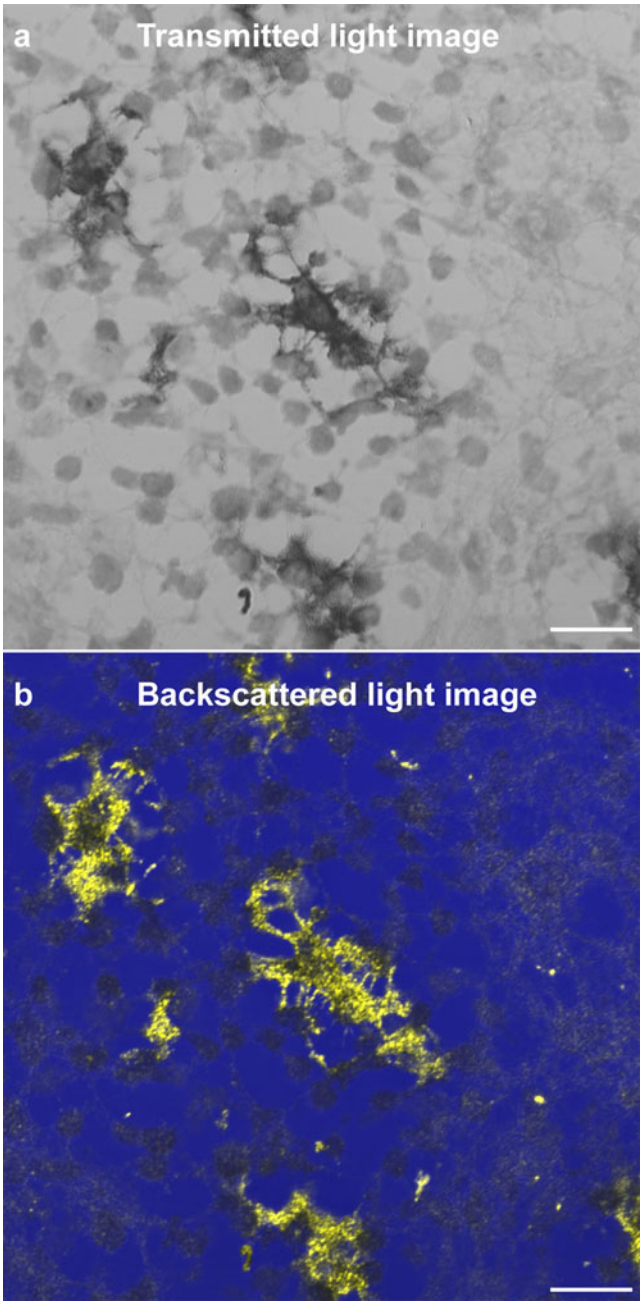

Fig. 9 Confocal backscattered light image of IgG-containing cells in the medulla of a 1-week-old chicken. **a** Conventional transmitted image of IgG-containing cells in the medulla. **b** Confocal backscattered light image in the medulla. In a transmitted light image, IgG was seen as a black shadow (**a**). In a confocal backscattered light image, backscattered light signals were visualized with yellow and IgG on IgG-containing cells produced strong backscattering signals. IgG was observed as accumulations of clusters. *Scale bars*=10 μm

distributions of IgG biosynthesizing cells in the bursa were different from those of IgG-containing cells after hatching (Fig. 5). It has been suspected that IgG-containing cells are IgG-biosynthesizing B cells (Kincade and Cooper 1971). However, this paper shows that IgG-containing cells in the medulla do not biosynthesize IgG after hatching. Our previous study also indicated that bursal B cells produce no or little IgG (Ekino et al. 1995). These results suggest that bursal B cells do not differentiate IgG-biosynthesizing cells in the medulla of the bursa in situ.

Nevertheless, IgG-biosynthesizing cells were found in the lamina propria and the cortex after hatching. These cells are postulated to be IgG-secreting plasma cells, because they did not express a B-cell marker (Fig. 8). In addition, IgG biosynthesizing cells are detected in chicken germinal centers containing T cells (Yasuda et al. 2002). T cells were distributed in germinal centers of the spleen, the lamina propria and the cortex of the bursa but not in the medulla of the bursa (Yasuda et al. 2002, Imamura et al. 2009). Thus, it is surmised that the absence of T cells prevents bursal B cells from differentiating into IgG-biosynthesizing cells or IgM-secreting cells in the medulla. Plasma cells were seen in the bursa of normal chickens, MIgG-free chicks and BDL-chickens after hatching (Fig. 6 and 10). Their origin is not clear at present. Probably, these plasma cells are derived from peripheral blood after hatching (Dent and Good 1965; Ekino et al. 1995).

In birds, reptiles and amphibians, IgY is the evolutionary ancestor of mammalian IgG and displays functional properties of mammalian IgG (Warr et al. 1995). Thus, chicken IgY has been perceived as the functional homolog of IgG. Transfer of a maternal antibody to offspring can be defined as the transfer of IgG or IgY by mother to offspring either through the placenta, colostrum, milk, or yolk (Brambell 1970). In chickens, IgG is transferred from hens to the chicks via the yolk (Kowalczyk et al. 1985; West et al. 2004). In the first step of the transfer, MIgG in the hen's serum is loaded into the yolk compartment of the developing oocyte (Morrison et al. 2001). In the second step, MIgG is transferred from the yolk to the embryonic circulation. The chicken yolk sac IgG receptor is the receptor responsible for the second step and the functional equivalent of the neonatal Fc receptor in mammals (West et al. 2004). The transfer of MIgG to the chick embryo is detected on day 7 of incubation and increases from day 15 of incubation. This transfer shows a dramatic increase during the last 3 days before hatching and the concentration of MIgG reaches its maximum around hatching (Kowalczyk et al. 1985). As a result, in newly hatched normal chickens, a substantial MIgG was distributed in the medulla and caused difficulties in distinguishing IgG-containing cells in the medulla (Fig. 11). Probably, MIgG is preferentially transported into the medulla from the capillaries, which are located outside the basement membrane (Fig. 1b). Washing with cold PBS prior to fixation removed most of MIgG in the medulla before hatching (Fig. 11).

Since we proposed a hypothesis that the attachment of MIgG to bursal cells generates IgG-containing cells in the

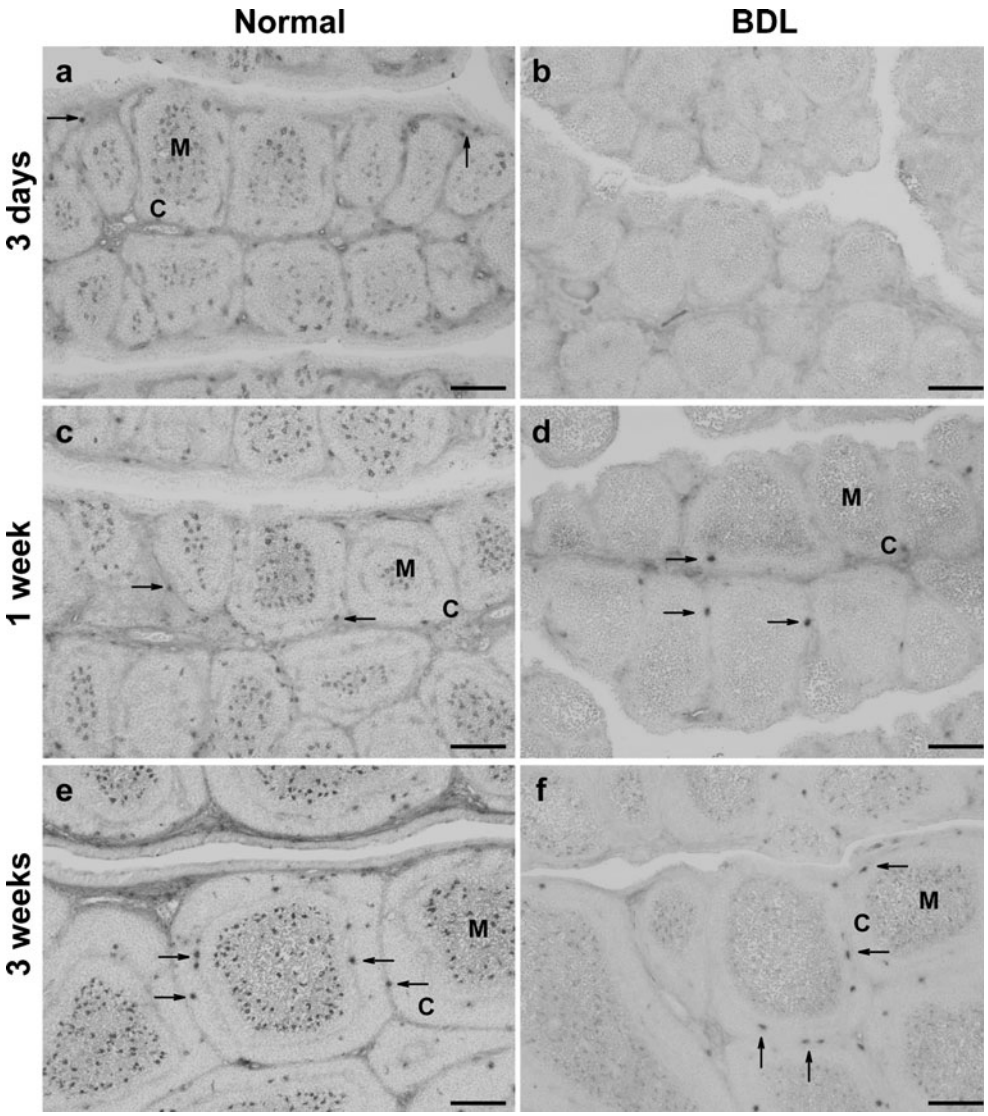

Fig. 10 Effects of BDL on the development of IgG-containing cells in the bursa 3 days (**a,b**), 1 week (**c,d**) and 3 weeks after hatching (**e,f**). In normal chickens, IgG-containing cells were always observed in the medulla, the cortex and the lamina propria of the bursa in normal chickens 3 days (**a**), 1 week (**c**), and 3 weeks (**e**) after hatching. *Arrows* indicate IgG-producing plasma cells in the cortex and the lamina propria of the bursa. In BDL-chickens, IgG-containing cells were not detected in the medulla at all ages tested (**b,d,f**), although a few IgG-producing plasma cells (*arrows*) were found in the cortex and the lamina propria of the bursa 1 week (**d**) and 3 weeks (**f**) after hatching. *Scale bars*=50 μm

medulla after hatching (Ekino et al. 1995), we established MIgG-free chicks from SBx-hens with agammaglobulinemia (Yasuda et al. 1998). In SBx-hens, the concentration of yolk IgG in fertilized eggs was almost negligible (0.002 mg/ml). Accordingly, newly hatched MIgG-free chicks did not possess MIgG in the serum. In MIgG-free chicks, IgG-containing cells were not observed in the medulla after hatching at all (Fig. 6). Therefore, it is demonstrated that IgG-containing cells in the medulla are induced by accumulations of MIgG after hatching. Furthermore, MIgG administration to MIgG-free chicks recovered the emergence of IgG-containing cells in the medulla after hatching (Fig. 7). Consequently, these results confirm that IgG-containing cells do not biosynthesize IgG in the medulla in situ and that they are generated by accumulations of MIgG in the medulla after hatching.

Histological examinations using CLSM show that IgG-containing cells in the medulla are about 20 μm in diameter and have dendrites (Figs. 7a, 8 and 9). They were observed as elongated cells, oval cells or round cells (Figs. 7a, 8d, f and 9). These two-dimensional sections of IgG-containing cells revealed that IgG-containing cells in the medulla are spindle-shaped cells with reticular branches and form a three-dimensional network in the medulla. Moreover, IgG-containing cells did not bear a B-cell marker. These results reveal that IgG-containing cells in the medulla are reticular cells. Before hatching, IgG showed “colloid-like” accumulations in the medulla (Figs. 4a, 7b and 11a,c). After hatching, however, IgG forms clusters or aggregates on reticular cells in the medulla (Fig. 9b). Consequently, IgG-containing cells in the medulla are reticular cells capturing aggregated MIgG. In addition, these histological features of IgG-containing reticular cells are similar to FDC (follicular dendritic cells) in germinal centers (Jeurissen et al. 1994) and the bursal secretory dendritic cells reported (Felföldi et al. 2005; Oláh and Vervelde 2008).

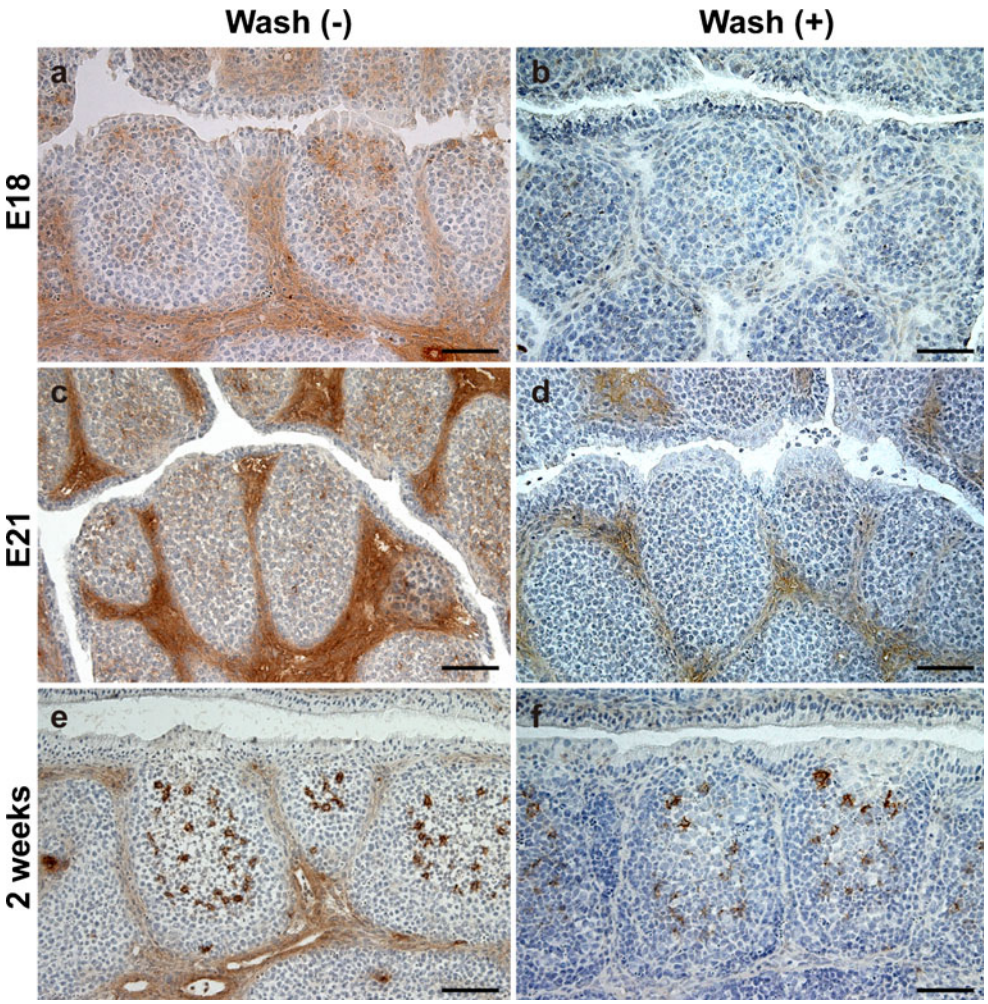

Fig. 11 Medullary localization of MIgG in the bursa of Fabricius. The bursal tissue was stained with anti-Cγ mAb on day 18 of incubation (*E18*) (**a, b**), on day 21 of incubation (*E21*) (**c,d**) and 1 week after hatching (**e,f**). Plenty of MIgG showed "colloid-like" accumulations in the medulla before hatching (Thorbecke et al. 1968; Kincade and Cooper 1971) (**a,c**). Cryostat sections (**b,d,f**) were washed with PBS prior to fixation. Most of the diffused MIgG was removed by washing prior to fixation (**b,d**). Aggregated MIgG in the medulla was kept even after washing (**f**). *Scale bars*=50 μm

The bursa of Fabricius is GALT in the form of a blind diverticulum connected to the cloaca and a major trapping site for environmental antigens (Ekino et al. 1985). Antigens from the cloaca are transported into the medulla across FAE (Schaffner et al. 1974; Sorvari et al. 1975; Sorvari and Sorvari 1977). It was suggested that external antigens in the medulla are involved in accumulations of MIgG on reticular cells in the medulla (Ekino et al. 1995). Then, we halted the influx of gut-derived antigens into the bursal lumen by BDL on day 18 of incubation and studied the effects of BDL on the development of IgG-containing cells in the medulla. BDL suppressed the emergence of aggregation of MIgG and IgG-containing cells in the medulla 3 days, 1 week and 3 weeks after hatching (Fig. 10). These results show that stimulations from the gut to the bursa are prerequisites for the accumulation of MIgG aggregates on reticular cells in the medulla after hatching. Presumably, the aggregation of MIgG is induced by conformational changes of MIgG after hatching (White et al. 1975; Klaus and Humphrey 1977; Klaus et al. 1980). So it is most likely that conformational changes of MIgG are induced by the formation of antigen–antibody complexes although antigen–antibody complexes in the bursal medulla have not been detected yet. In fact, complement activation plays a key role in dendritic localization of aggregated IgG (White et al. 1975; Klaus and Humphrey 1977; Klaus et al. 1980). Complement and mannan-binding proteins, which are provided to chickens around hatching (Gabrielsen et al. 1973; Laursen et al. 1998; Laursen and Nielsen 2000), may be activated by bacterial antigens and contribute to the dendritic distribution of aggregated MIgG in the medulla after hatching. The data presented here suggest that environmental antigens form immune complexes with MIgG in the medulla (Ekino et al. 1995, Arakawa et al. 2002), which are subsequently captured on the surface of reticular cells mediated either by Fcγ receptor or complement receptor (Viertlboeck et al. 2007; Thunold et al. 1981).

The bursa of Fabricius is known as a primary lymphoid organ or a mutant breeding organ (Jerne, 1971), which provides the preimmune B cell repertoire. An almost infinitive number of antibody specificities are diversified by the somatic mutation in the bursa (Reynaud et al. 1994). However, it is not clear how B-cell mutants are selected and how the functional B-cell repertoire is established in the bursa.

The implication of present data is that environmental antigens with MIgG on reticular cells play a key role in the selection of B-cell mutants in the bursa and contribute to the establishment of the functional B-cell repertoire after hatching (Ekino et al. 1995, Arakawa et al. 2002).

In our previous study, dendritic distribution of aggregated IgG is detected in lymph follicles of calf ileal Peyer's patch after birth (Yasuda et al. 2002), which is also a primary lymphoid organ of B-cell diversification caused by somatic mutation (Parng et al. 1996). Presumably, aggregated IgG in ileal Peyer's patches of calf also provide a similar function to that in the bursa of Fabricius of chickens.

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