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The significance of intestinal flow in the maturing of B lymphocytes and the chicken antibody response

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INTRODUCTION

It has already been widely demonstrated that the bursa of Fabricius plays the role of a primary organ in the development of B lymphocytes in birds. It has been shown by means of surgical bursectomy (Glick, Chang & Jaap, 1956; Mueller, Wolfe & Meyer, 1960; Peterson, Cooper & Good, 1965) and pharmacological bursectomy (Mueller *et al.* 1960; Peterson *et al.* 1965; Toivanen, Toivanen, Linna & Good, 1972) that the removal of the bursa produces considerable alterations to the thymus-independent system and that only bursal cells are capable of reintegrating the immune functions in animals without a bursa (Toivanen *et al.* 1972; Ratcliffe *et al.* 1987). Some authors have concentrated their attention on the influence that stimuli from the intestinal lumen might have on the maturing of B lymphocytes; in particular, some of them believe that the differentiation of B cells in the bursa of Fabricius takes place as a completely antigen-independent process (Lydyard, Grossi & Cooper, 1976; Moriya & Ichikawa, 1979; Bockman, 1979). By contrast, other authors affirm that antigen stimulation may play some kind of role in favouring the complete development of B cells (Thompson & Cooper, 1971; Schaffner, Hess & Cottier, 1974*b*; Ekino *et al.* 1979), or at least that it may influence the amplification of the lymphocyte populations that normally develop in the bursa of Fabricius (Ekino *et al.* 1980; Boyd, Wilson, Mitrangas & Ward, 1987). A study was conducted by isolating the bursa of Fabricius from the intestinal flow in chicks at their 16th day of embryo life and at hatching (Lupetti & Dolfi, 1980*a*). Lymphopoiesis still takes place under these conditions, with the formation of bursal lymphatic follicles that are differentiated into a medulla and a cortex over a period of observation including the first 15 days of life (Lupetti & Dolfi, 1980*b*). On the basis of these findings, it is natural to wonder whether lymphocytes from the bursa of Fabricius isolated from the cloaca, which have therefore not come into contact with intestinal material, can still reach complete functional maturity. In order to answer this question, we compared the distribution of B lymphocytes in the bursa of Fabricius, and the antibody responses to sheep red blood cells (SRBC), of three groups of animals which were, respectively, subjected to isolation of the bursa from the intestinal flow, to a sham operation and to no operation at all. As it has been shown that a fair number of B lymphocytes move into the area of diffuse lymphoid infiltration of the bursa and of the cloaca (Dolfi, Bianchi & Lupetti, 1988), the present study also investigated the distribution of B lymphocytes in the dorsal wall of the cloaca in 20 days old chicks, after ligating the bursa of Fabricius at hatching.

MATERIALS AND METHODS

Experimental groups

Fertile eggs of the Hubbard strain were obtained from a local hatchery and were incubated in our laboratories. All the chicks received food and water *ad libitum*. 42 chicks were divided up into three groups of 14 animals each: the first group was formed of chicks whose burso-cloacal stalk was ligated at hatching, the second included chicks subjected to a sham operation, and the third was made up of unoperated chicks. Five 8 weeks old chickens were used for the preparation of chicken Ig and two rabbits were employed for the production of the anti-chicken Ig antiserum. Seven 30 days old chicks were used to characterise the anti-chicken Ig antiserum by means of indirect immunofluorescence.

Surgical techniques

The environment and the instruments used were sterilised; anaesthesia was produced by local injection of 0·25 ml of 2% Lidocaine. All animals were operated on within 12 hours of hatching.

Bursal ligature

The technique followed consisted of the ligature and the resection of the burso-cloacal stalk (Bl animals), as already described in a previous paper of ours (Lupetti & Dolfi, 1980*a*).

Sham operations

An incision was made on the skin, and the bursa was visualised, as in the ligature operation; however, we then proceeded directly to the suture of the wall.

Immunisation and sampling procedures

The three experimental groups were used to study the antibody response to SRBC. Each animal belonging to a first group of 21 16 days old chicks received intravenously 10^8 SRBC suspended in 0·25 ml of phosphate-buffered saline. Blood samples were taken four days later in order to estimate anti-SRBC agglutinin. The same immunisation procedures were repeated with a second group of 21 chickens of the age of eight weeks, in order to evaluate the primary response at a later period (see Table 1).

The sera were inactivated before use at 56 °C for 30 minutes, and were centrifuged at 3000 rpm for 20 minutes. Testing for circulating anti-SRBC antibodies was carried out by standard agglutination procedures, using the same antigen employed for immunisation. Results are expressed as the geometric mean (Log -2) of the antibody titre $\pm$ S.E. The statistical significance of differences was evaluated by Student's t test.

Preparation and characterisation of the anti-chicken Ig antiserum

Blood samples were taken by cardiac puncture from five 8 weeks old chickens immunised against SRBC and the serum was obtained by centrifuging at 3000 rpm for half an hour. Subsequently, the serum was decomplemented at 56 °C for a further 30 minutes and filtered. A small sample of serum was employed to evaluate the response to SRBC, which was high in all animals. The serum albumin was then precipitated using 25% sodium sulphate and dialysed against a borated buffer solution at pH 8·2 (Benedict, Larson & Homoyoun, 1963). The globulins that remained after this treatment were further fractioned by means of preparatory electrofocusing; this was

Table 1. *Treatment schedule*

Experimental group		Number of chicks	Treatment at age (days)				
			0	16	20	56	60
N	20 days	7	—	iv	k	—	—
N	60 days	7	—	—	—	iv	k
Bl	20 days	7	L	iv	k	—	—
Bl	60 days	7	L	—	—	iv	k

N, unoperated chick; Bl, chick whose burso-cloacal stalk was ligated; L, ligature operation; iv, intravenous injection of SRBC; k, killing.

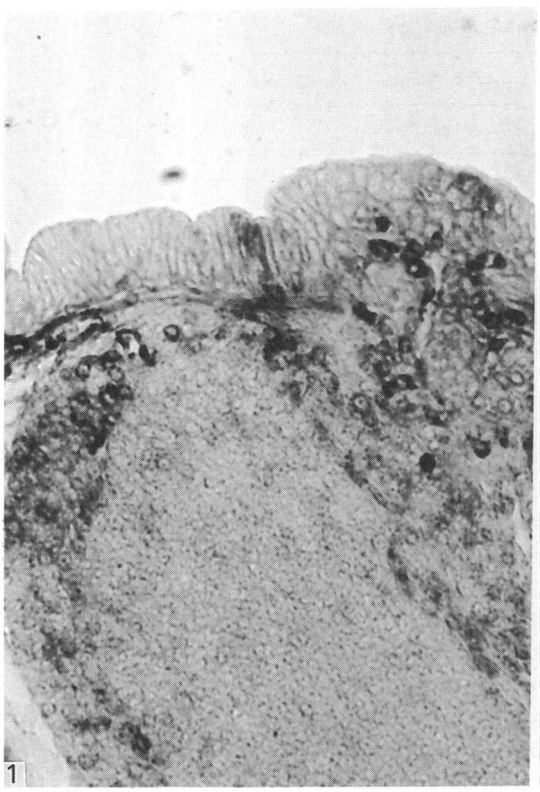
carried out in a range of pH 5–8·5, with an initial tension of 800 volts in the column, for a duration of 36 hours. The eluate was collected in one hundred 1 ml fractions, using a fraction-collector; each fraction was read on the spectrophotometer at 280 nanometers in order to measure the maximum protein concentrations. Three peaks were found, only one of which possessed SRBC-agglutinating properties. The fractions with this immunological characteristic were collected together and the solution obtained was injected subcutaneously into two rabbits, together with Freund's adjuvant, in two successive injections at an interval of 15 days. After a third intravenous injection, the animal with the highest level of antibodies was bled to death. The serum obtained by centrifuging was decomplemented and precipitated in ammonium sulphate. The rabbit Ig thus obtained was studied by means of immunodiffusion against the whole chicken serum and revealed two precipitation arcs corresponding to the chicken Ig. In order to characterise further the antiserum, an immunofluorescence study was carried out on cell suspensions obtained from peripheral blood; using an indirect sandwich immunofluorescence method, morphological pictures were obtained corresponding to those already described for antibody-producing cells. Positive cells in blood and in the spleen were also counted, and Ig-producing cells were found to be respectively 24% and 17% of the cells observed.

Immunoperoxidase technique

The bursa of Fabricius, together with the burso-cloacal stalk and the cloaca, was removed under ether anaesthesia from chicks belonging to each of the three groups at ages of 20 and 60 days; they were fixed in Bouin's solution, embedded in paraffin, and sectioned at a thickness of 7 μm. After removal of the paraffin, the sections were treated for immunoperoxidase using the Ortho Diagnostic System kit; the antiserum obtained against chicken Ig in the rabbit was used as the first antiserum. The method followed was the standard one for the PAP system (peroxidase–antiperoxidase).

Positive cell count

21 sections of 7 μm each were observed for every age group and positive cells were counted with a $\times 10$ ocular with a grid divided into squares and a $\times 40$ lens. Twenty five microscopic fields were observed for each section at the level of the medulla and 25 at the level of the cortex of the bursal follicle, while 50 fields of the same number of sections were observed at the point of cloacal lymphoid infiltration. Counts were made in randomly chosen fields. The data obtained were expressed as the mean percentage of the total number of cells per field, taken arbitrarily as equal to 100.



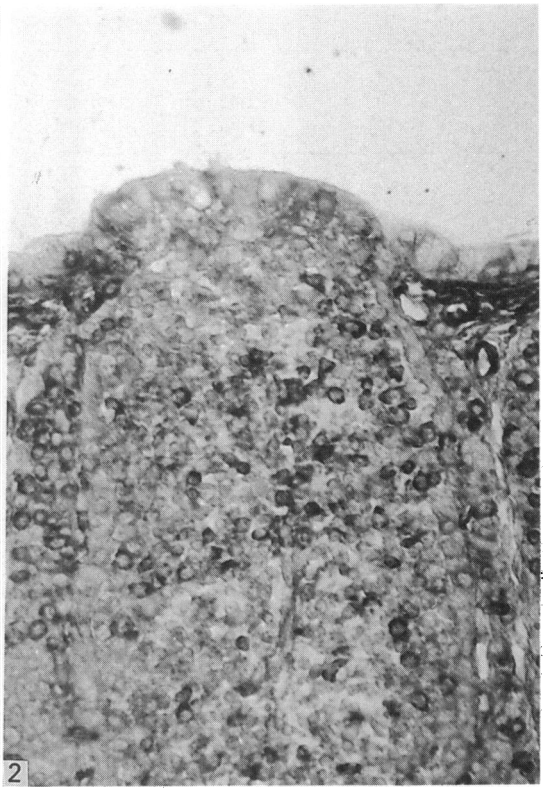


Fig. 1. Unoperated 20 days old chick. The cells that are positive to the Ig antiserum reaction are concentrated particularly in the cortical component of the follicle. Bouin, paraffin, immunoperoxidase. × 335.

Fig. 2. Twenty days old chick whose burso-cloacal stalk was ligated at hatching. The reactive cells are fairly widespread and are situated both in the cortex and in the medulla of the bursal follicle. Bouin, paraffin, immunoperoxidase. × 335.

RESULTS

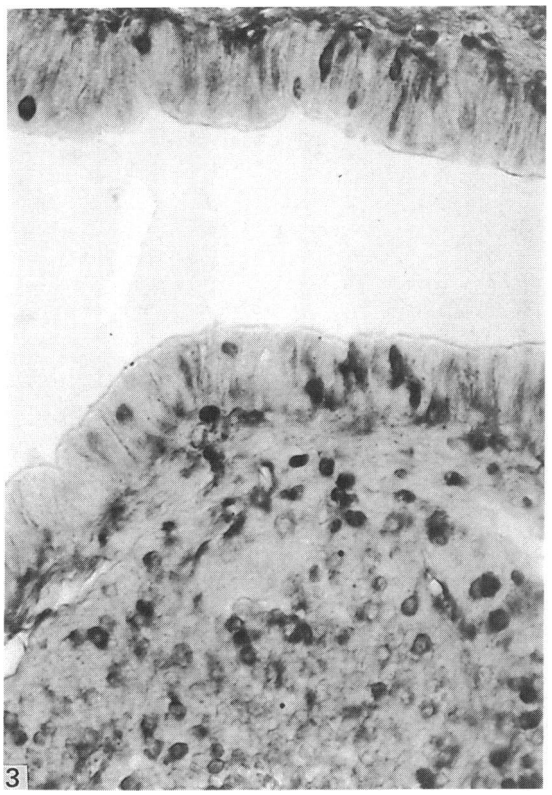
Distribution of B lymphocytes in the bursal follicles

Unoperated chicks

The picture obtained at the age of 20 days with anti-Ig immunoperoxidase reveals the highest concentration of B lymphocytes in the cortical components of the bursal follicles. By contrast, the medullary part appears to be almost completely devoid of reactive cells. Some cells that are positive for the reaction can be seen also at the level of the covering epithelium of the bursal plicae (Fig. 1). The mean proportion of reactive lymphocytes was, respectively, 21·52% in the cortex and 1·00% in the medulla. The histological picture is different at the age of 60 days, since the Ig-reactive cells are present not only in the cortical component but can also be found in fairly large quantities in the medulla (Fig. 3); the mean values obtained from the cell count were 22·68% in the cortex and 7·24% in the medulla.

Chicks whose bursa was ligated at hatching

The histological picture observed at the age of 20 days in this group of animals reveals the presence of B cells in fairly large numbers, both in the cortical component (mean 28·20%) and in the medullary component (mean 18·08%). Lymphocytes that



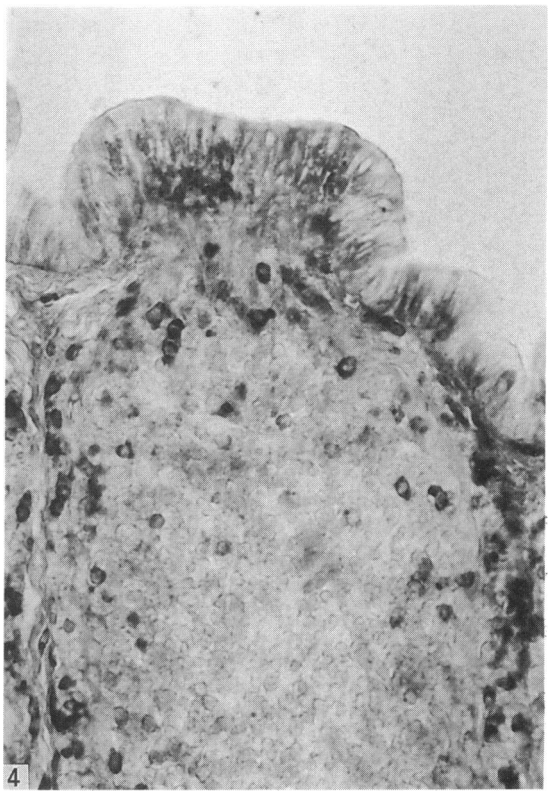


Fig. 3. Unoperated 60 days old chicken. A positive reaction is found in cells situated in both areas of the follicle; the positive cells included in the epithelial region are also numerous. Bouin, paraffin, immunoperoxidase. × 335.

Fig. 4. Sixty days old chicken whose burso-cloacal stalk was ligated at hatching. In this case, the positive cells are more abundant in the cortical component, in the interfollicular connective tissue and in the epithelium. Bouin, paraffin, immunoperoxidase. × 335.

have infiltrated the covering epithelium and the interfollicular connective tissue also appear to be numerous (Fig. 2).

In the 60 days old chicken, the distribution of B lymphocytes is more highly concentrated in the cortical component of the follicle, even though some reactive cells are present in the medulla. B lymphocytes appear to be numerous in the interfollicular connective tissue (Fig. 4). The mean percentages found were 20·40 % in the cortex and 3·04 % in the medulla.

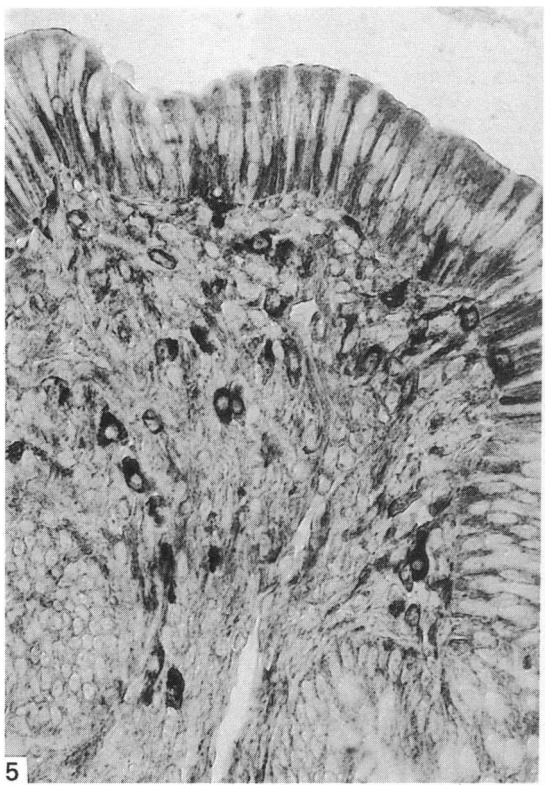
Animals subjected to a sham operation

The histological pictures and the percentages of reactive cells in this group of animals were practically identical, at the age of both 20 and 60 days, to those found for the group of unoperated animals; the variations observed have no statistical significance.

Distribution of B lymphocytes in the cloaca

Unoperated chicks

At the age of 20 days, B lymphocytes are present at the level of the cloaca in the tunica propria and also in the covering epithelium (Fig. 5). Although not particularly numerous, the reactive cells reach a mean of 13·16 %.



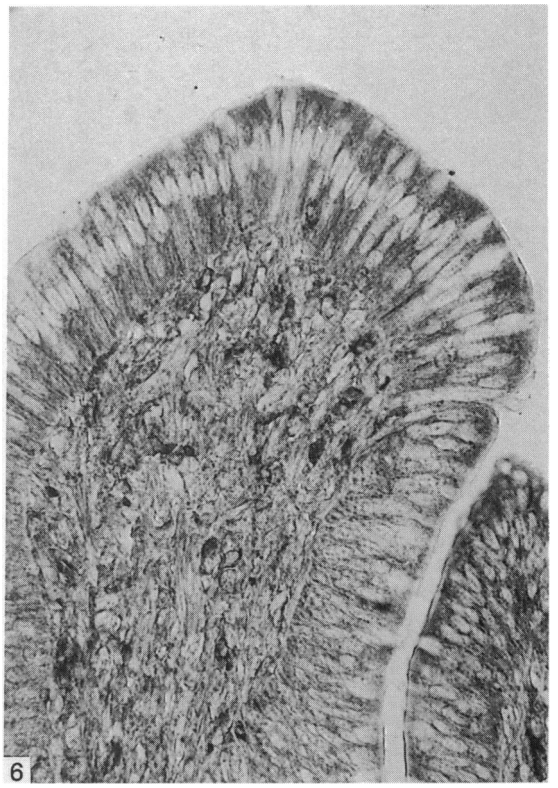


Fig. 5. Cloaca of an unoperated 20 days old chick. The tunica propria appears to be infiltrated by numerous cells that are positive to the anti-Ig reaction. Bouin, paraffin, immunoperoxidase. ×455.

Fig. 6. Cloaca of a 20 days old chick whose burso-cloacal stalk was ligated at hatching. Cells that are positive for the reaction are very scarce. Bouin, paraffin, immunoperoxidase. ×455.

Chicks with the bursa ligated

The cloaca sections from 20 days old chicks present a picture in which reactive B cells are extremely scarce both in the tunica propria and in the epithelium (Fig. 6). The mean percentage of positive cells was 2·08 %.

SRBC antibody response

Unoperated chicks

The mean agglutinin level in the group of unoperated chicks was $5{\cdot}14 \pm 0{\cdot}55$ at the age of 20 days and this figure went up to $8{\cdot}71 \pm 0{\cdot}64$ in 60 days old chicks.

Chicks with a ligated bursa

Samples from 20 days old animals showed a mean agglutinin value of $8{\cdot}00 \pm 0{\cdot}68$. This figure differed significantly ($P < 0{\cdot}01$) from the levels found in unoperated chicks of the same age. By contrast, at the age of 60 days, the mean anti-SRBC agglutinin level was practically identical to that of unoperated controls ($8{\cdot}67 \pm 0{\cdot}8$).

Chicks subjected to a sham operation

The mean values found for this group of animals were $6{\cdot}1 \pm 0{\cdot}25$ at the age of 20 days, and $8{\cdot}5 \pm 0{\cdot}5$ at the age of 60 days.

The immunological results are summarised in Table 2.

Table 2. *Summary of immunological results*

Experimental group		Number of chicks	Mean anti-SRBC agglutinin at age (days)	
			20	60
N	20 days	7	5·14±0·55	—
N	60 days	7	—	8·71±0·64
Bl	20 days	7	8·00±0·68	—
Bl	60 days	7	—	8·67±0·80
SO	20 days	7	6·10±0·25	—
SO	60 days	7	—	8·50±0·50

N, unoperated chick; Bl, chick subjected to ligature of the burso-cloacal stalk; SO, chick subjected to sham operation.

DISCUSSION

The results obtained in the present study indicate that the differentiation of B lymphocytes still takes place even without any stimulus from the intestinal flow and that ligature of the burso-cloacal stalk at hatching, with the consequent elimination of any stimulus from intestinal flow, leads to an increase in B lymphocytes in the bursa at the age of 20 days, together with an increase in the response to SRBC compared with unoperated controls. At a later age, these two parameters tend to return to normal levels. Ligature not only eliminates the intestinal stimuli at the bursal level but, at the same time, interrupts the migration of lymphocytes from the bursa to the areas of cloacal lymphoid infiltration.

Ever since the bursa of Fabricius was recognised as being the primary organ in the differentiation of B lymphocytes, one of the main themes of research has been to understand whether the maturing of these cells is completely antigen-independent or whether antigens may be involved in some way in this phenomenon. As regards the role played by stimuli from intestinal material, this was investigated by Thompson & Cooper (1971), who introduced bursal and thymic fragments into the peritoneal cavity and noticed a normal development of the thymic lymphoid tissue and a failure to mature of the bursal lymphoid tissue. They consequently concluded that intestinal material might in some way influence lymphopoiesis and the differentiation of B lymphocytes. When, in 1971, Bockman and Cooper described the epithelial cells associated with lymphatic follicles (FAE), greater emphasis was placed on the role played by intestinal factors, since FAE cells were found to be capable of absorbing various kinds of material, and transferring it to the medulla of the bursal follicle (Bockman & Cooper, 1973; Schaffner *et al.* 1974*a*; Schaffner, Hess & Cottier, 1974*b*; Gilmore & Bridges, 1977; Naukkarinen, Arstila & Sorvari, 1978; Beezhold, Sachs & Van Alten, 1983). At this time it was noted that after the early phases of development, in which it functions as the primary organ for the differentiation of B lymphocytes, the bursa of Fabricius develops the capacity, and carries out the functions, of a secondary lymphoid organ associated with the intestine (Van Alten & Meuwissen, 1972; Toivanen *et al.* 1972; Sorvari & Sorvari, 1977; Hirota, Vainio & Toivanen, 1981; Lupetti, Dolfi, Malatesta & Michelucci, 1984; Ekino *et al.* 1985). The specific problem of the actual role played by intestinal material in the function of the bursa, first as a primary organ and later as a secondary one, was investigated in experiments interrupting communication between the bursa and the cloaca (Lupetti & Dolfi, 1978;

Lupetti & Dolfi, 1980*a*, *b*; Ekino *et al.* 1980). In order to test the influence of intestinal contents on bursal lymphopoiesis, the bursal stalk was surgically removed after double ligature with Ethicon thread (Lupetti & Dolfi, 1980*a*), carried out on the 16th day of embryonic life or at hatching. In this way, contact between the bursa of Fabricius and the intestinal flow was avoided in embryos before the opening of the urodeal membrane, while in chicks operated at hatching, bacterial contamination of the bursa was avoided. This operation made it possible to safeguard the bursal vessels which begin their course near the connection between the bursa and the burso-cloacal stalk. Observations carried out at the 15th day of life did not reveal any changes in the bursal structures compared with unoperated controls. It thus appeared that the integrity of the anatomical relationship between the bursa and the cloaca was not necessary for normal lymphopoiesis, though a normal vascularisation is, on the contrary, indispensable (Lupetti & Dolfi, 1980*b*). The findings of Ekino *et al.* (1980) and Ekino (1988) indicate that ligature of the bursal stalk determines the abolition of B cell proliferation, the failure of IgG and IgA to develop in the follicular medulla and a reduction in the response to SRBC. These findings would appear to confirm a considerable participation of antigenic stimuli in the maturing of the immune B system in the chicken, though the same author suggests that intestinal stimuli only have the role of accelerating the development of cells forming specific antibodies. The difference between these results and ours may be due to the different microsurgical technique employed. The present findings indicate that both the number of the lymphocytes and the response to SRBC are higher in chicks with a ligated bursa than in unoperated chicks three weeks after the operation carried out at hatching. An increase in the response to SRBC had already been found in another experiment (Lupetti *et al.* 1984). This would appear to be consistent with the high levels of B cells found after three weeks of life in chicks with a bursa ligated at hatching. As regards the increase in B lymphocytes, we believe that a functional mechanism is involved which is in need of further explanation. Probably, the B lymphocytes that are differentiated in the bursa of Fabricius undergo a systemic peripheralising by the haematic route and a local peripheralising by the intramural route. Recent studies have shown that the areas of bursal and cloacal infiltration are defensive barriers where large numbers of B lymphocytes are present, which increase with age. In these areas of diffuse lymphoid infiltration where contact with the antigen is massive, B cells infiltrate the tunica propria and the epithelium in large quantities, and pass into the lumen. They probably play a role in local defence and modulation of the entry of antigens that are already recognised into the bursa of Fabricius (Dolfi *et al.* 1988). The ligature of the burso-cloacal stalk might cause a block in the intramural peripheralisation route, with the result that B lymphocytes would first be absorbed into the bursal follicle, and subsequently would follow the route of central peripheralisation in massive quantities. This might explain the increase in the response to SRBC. Confirmation of this would appear to be offered by the results obtained in Bl animals, whose cloacae appear to be practically devoid of any B lymphocytes. Results obtained in the bursae of 60 days old animals do not seem to show any significant differences in the total number of reactive cells; a different distribution of positive cells can be seen, however, in animals whose bursa was ligated at hatching, compared with unoperated controls. In operated animals, the Ig-producing cells are more highly represented in the cortical component of the bursal follicle while in unoperated animals they appear to be more numerous in the medullary component. The behaviour observed in the unoperated chick would appear to be in agreement with the fact that the bursa acquires more and more the function of a secondary lymphoid organ; it would thus appear logical that a greater

quantity of Ig-positive lymphocytes should be present in the bursal follicle and these tend to infiltrate the epithelium. It is more difficult to interpret the picture observed after ligature. The lack of intestinal antigens in the ligated bursa might be the cause of a reduction in the number of positive lymphocytes. In chicks whose bursa has been ligated for 60 days, it is common to find the accumulation of a fairly large quantity of a viscous liquid in the bursal lumen, with a consequent compression of the bursal structures. This compression might be wholly or partly responsible for a reduced functional activity of the bursal follicle, with a reduction in medullary B lymphocytes. In conclusion, the present experiment underlines the independence from the antigenic stimulation of the maturing of B lymphocytes. Generally speaking, it would appear more plausible to postulate that intestinal stimuli play a role of modulating the proliferative phenomena that the immune system cells undergo as the bursa gradually acquires the functions of a secondary lymphoid organ.

SUMMARY

The aim of the present study was to evaluate the influence of intestinal material on the maturing of B lymphocytes in the bursa of Fabricius and on the antibody response to SRBC. Experiments were carried out on chicks whose burso-cloacal stalk had been ligated at hatching, in order to avoid any contact between the bursal tissues and intestinal material. Chicks subjected to a sham operation, or not operated at all, were used as controls. Results obtained by immunoperoxidase test, using an anti-chicken Ig antiserum, indicate that burso-cloacal stalk ligature leads to an increase in B cells in the bursa of Fabricius 20 days after the operation and a slight decrease after 60 days. The response to SRBC reveals an increase after 20 days, though no variations are found with respect to controls after 60 days. Thus, B lymphocytes mature independently of intestinal stimuli. Furthermore, observations carried out at the age of 20 days indicate that B lymphocytes disappear almost completely from the area of cloacal lymphoid infiltration in chicks with a ligated bursa. It is hypothesised that together with a systemic peripheralisation of B lymphocytes in secondary lymphoid organs and in blood, there exists an intramural local peripheralisation, which is interrupted by ligature of the burso-cloacal stalk.

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