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Humoral immune responses characteristic of testosterone–propionate-treated chickens

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Summary. White Leghorn chickens treated with testosterone–propionate on the 3rd day of embryonation were immunized with a mixture of sheep red blood cells, *Brucella abortus* and *Salmonella pullorum* at various ages, and the resulting agglutinins were titrated. The production of IgM antibody against sheep red blood cells was not affected significantly by testosterone–propionate. On the contrary, immune responses against the bacterial antigens were strongly suppressed by the same treatment. Production of IgG antibodies was strongly suppressed by the same treatment. There was little correlation between the production of IgM antibody against sheep red blood cells and the presence of bursal follicles. Immune responses against bacterial antigens correlated with the presence of the follicles. Production of IgG antibodies also correlated with the maintenance of bursal lymphoid structure.

INTRODUCTION

Warner, Uhr, Thorbecke and Ovary (1969) reported that the degree of suppression of the immune response by hormonal bursectomy varied with individual birds. Lerner, Glick and McDuffie (1971) observed that the 2-mercaptoethanol (2-ME) sensitive agglutinin response to sheep red blood cells (SRBC) by hormonally bursectomized chickens was normal, although production of antibody of IgG type was decreased.

In the present paper we show that the antibody response by testosterone–propionate (TP) treated chickens is characterized by preferential production of 2-ME-sensitive agglutinins to SRBC, and by suppression of agglutinin responses to bacterial antigens, and of antibody responses of IgG type. It also shows that the latter immune responses correlated with the maintenance of bursal lymphoid structure.

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MATERIALS AND METHODS

Chickens

Chickens used were White Leghorn Hy-Line (Takeuchi Hatchery Incorporated, Osaka) and inbred strain Anthony from our own flocks.

Treatment of chickens

TP was purchased from Sigma Chemical Company, St Louis, Missouri. Hormonal bursectomy was performed by dipping 3-day-old embryonated eggs into 2·0 per cent TP ethanol solution for 5 s. Such hormonally bursectomized chickens were designated as TP3. On 1 and 7 days after hatching, these chickens were examined for remnants of the bursa of Fabricius. Bursal remnants were removed surgically at

hatching or at 7 days of age. These chickens were designated as 0TP3 and 7TP3 respectively. Untreated chickens (Con-H and Con-A) were used as controls.

Antigens

The antigens used were SRBC, *Brucella abortus* (BA) and *Salmonella pullorum* (SP) strain 4054. SRBC were stabilized in Alsever's solution at 4° for less than 1 week. BA cells were purchased from the National Institute for Animal Health. Cells of SP were harvested from 18 h growth on YCC agar (Ishii, Sakazaki and Ushiko, 1958), inactivated by treating with 0·1 per cent formalin in phosphate-buffered saline (PBS) and washed twice with PBS.

Immunization

A mixture of 2×10^9 SRBC and 1 (expts 1 and 2) or 5 mg (wet weight) (expts 3, 4, 5 and 6) each of BA and SP cells was injected intravenously into each chicken of the TP-treated and control groups at indicated ages.

Antibody titration

Bleedings were performed 7 days after the first and the second stimulation. Agglutinins to SRBC were measured by microtitration with 25-μl volumes of the serum to be titrated in 2-fold dilutions and of 0·5 per cent SRBC. PBS was used as diluent. The plates were incubated at 37° for 1 h. Agglutinins to BA and

Table 1. Immune responses to sheep red blood cells in testosterone–propionate-treated chickens

Expt no.	Groups	Primary response				Secondary response			
		No treatment*		2-ME treatment†		No treatment		2-ME-treatment	
		Responders‡	Titre§	Responders	Titre	Responders	Titre	Responders	Titre
Immunizations at 3·5 and 5·5 weeks of age				‡					
1	TP3-H	5/5	2·4±1·9	0/5		4/4	7·5±0·9	2/4	3·5±0·7
	0TP3-H	1/4	3·0	0/4		3/3	3·2±2·5	0/3	
	Con-H	9/9	3·6±1·3	0/9		9/9	6·9±0·5	8/9	3·4±1·2
2	TP3-A	9/12	2·3±1·3	0/12		9/9	5·3±1·9	3/9	1·8±1·0
	Con-A	5/5	2·7±0·7	0/5		5/5	5·6±0·4	4/5	3·7±0·8
Immunizations at 4 and 6 weeks of age									
3	TP3-H	3/6	2·3±0·4	0/6		4/5	7·0±1·4	3/5	3·3±1·2
	0TP3-H	1/7	2·0	0/7		3/4	3·5±0·7	0/4	
	Con-H	4/4	3·8±0·3	4/4	1·4±0·5	4/4	7·3±1·7	4/4	4·5±0·8
4	TP3-A	2/8	2·0±1·4	0/8		4/7	5·4±2·6	3/7	1·8±1·2
	0TP3-A	1/6	1·0	0/6		4/6	1·6±1·0	0/6	
	Con-A	3/3	2·7±0·6	3/3	1·3±0·8	3/3	6·0±0·9	3/3	3·8±0·8
Immunizations at 6 and 9 weeks of age									
5¶	TP3-H	4/6	4·1±1·3	1/6	1·0	5/6	5·7±1·4	5/6	1·5±1·8
	0TP3-H	0/3		0/3		1/3	1·0	0/3	
	7TP3-H	0/3		0/3		1/3	1·0	0/3	
	Con-H	5/5	5·6±2·8	5/5	1·8±0·7	5/5	6·3±2·3	5/5	4·2±1·9
6**	TP3-H	6/7	4·7±0·3	2/7	1·0	7/7	5·5±0·6	5/7	1·5±0·2
	Con-H	5/5	5·8±1·3	2/5	1·0	5/5	5·5±2·2	5/5	3·8±1·4

Control and TP-treated chickens were immunized intravenously at indicated ages with a mixture of SRBC, BA and SP antigens. Agglutinins were determined in sera taken a week after both the first and second stimulations.

* Titres in sera not treated with 2-ME.

† Titres in sera treated with 2-ME.

‡ Number of responding chickens per number of total chickens.

§ Mean titres of responders and standard deviation.

¶ Each antigen was injected into individual chickens.

** Antibody titrations of 0TP3-H and 7TP3-H groups were not done.

SP were titrated by a technique similar to that used for determination of SRBC agglutinins; 0·2 per cent suspension of BA and SP cells (wet weight/volume) was used. After addition of each antigen mentioned above the plates were incubated at 37° for 1 h and at 4° for 24 h. The titre was expressed in $\log_2$ of the reciprocal of the highest dilution giving complete or incomplete agglutination. Incomplete agglutination was taken as 0·5 in $\log_2$. Number of responders in each group and mean ± s.d. $\log_2$ titre of responders are given in the tables.

Antibody titration after 2-ME treatment

In our preliminary experiments, the treatment with 2-ME showed that the antibody activities of macroglobulin fractionated by analytical ultracentrifugation and chromatography on Sephadex G-200 were sensitive to reduction by 2-ME. The serum was added with an equal volume of 0·2 M 2-ME; the mixture was allowed to stand for 40 min at 37°. The sera treated with 2-ME were titrated by a method similar to that mentioned above for determination of agglutinins to SRBC, BA or SP.

Microscopic examination

Seven days after immunizations with a mixed antigen at 3·5 and 5·5 weeks of age, bursal remnants were taken from TP-treated and control chickens and

Table 2. Immune responses to *Brucella abortus* in testosterone–propionate-treated chickens

Expt no.	Groups	Primary response				Secondary response			
		No treatment*		2-ME treatment†		No treatment		2-ME treatment	
		Responders‡	Titre§	Responders	Titre	Responders	Titre	Responders	Titre
Immunizations at 3·5 and 5·5 weeks of age									
1	TP3-H	1/5	5·5	0/5		1/4	5·0	1/4	2·0
	0TP3-H	0/4		0/4		0/3		0/3	
	Con-H	9/9	4·7 ± 1·9	0/9		9/9	5·3 ± 1·0	8/9	3·0 ± 1·2
2	TP3-A	0/12		0/12		0/9		0/9	
	Con-A	5/5	3·1 ± 0·9	3/5	1·3 ± 0·6	5/5	4·5 ± 0·8	5/5	1·8 ± 0·8
Immunizations at 4 and 6 weeks of age									
3	TP3-H	1/6	1·0	0/6		4/5	5·5 ± 0·3	1/5	1·0
	0TP3-H	0/7		0/7		2/4	1·5 ± 0·7	0/4	
	Con-H	4/4	5·5 ± 0·6	4/4	1·4 ± 0·4	4/4	7·7 ± 0·6	4/4	3·5 ± 0·6
4	TP3-A	2/8	3·3 ± 0·4	0/8		4/7	5·4 ± 0·3	2/7	0·8 ± 0·8
	0TP3-A	1/6	1·0	0/6		0/6		0/6	
	Con-A	3/3	5·3 ± 0·4	3/3	1·3 ± 0·6	3/3	7·7 ± 3·5	3/3	3·8 ± 0·5
Immunizations at 6 and 9 weeks of age									
5¶	TP3-H	0/5		0/5		3/5	2·8 ± 0·4	0/5	
	0TP3-H	0/3		0/3		0/3		0/3	
	7TP3-H	0/3		0/3		0/3		0/3	
	Con-H	5/5	6·5 ± 2·2	1/5	1·0	4/4	3·5 ± 1·5	4/4	4·2 ± 2·4
6	TP3-H	2/6	1·5 ± 0·3	0/6		4/6	4·1 ± 1·5	2/6	1·0
	0TP3-H	0/5		0/5		1/4	1·0	0/4	
	7TP3-H	0/5		0/5		0/3		0/3	
	Con-H	5/5	5·4 ± 0·1	3/5	1·6 ± 0·5	5/5	7·5 ± 0·4	5/5	1·8 ± 0·4

Control and TP-treated chickens were immunized intravenously at indicated ages with a mixture of SRBC, BA and SP antigens. Agglutinins were determined in sera taken a week after both the first and second stimulations.

* Titres in sera not treated with 2-ME.

† Titres in sera treated with 2-ME.

‡ Number of responding chickens per number of total chickens.

§ Mean titres of responders and standard deviation.

¶ Each antigen was injected into individual chickens.

fixed with 10 per cent formalin. Eight to thirteen sections were prepared from each remnant, and stained with haematoxylin and eosin (H & E).

RESULTS

Immune responses to sheep red blood cells, *Brucella abortus* and *Salmonella pullorum*, antigens in testosterone–propionate-treated chickens

White Leghorn chickens were hormonally bursectomized on the third day of embryonation and immunized with a mixed antigen at indicated ages. Serum samples taken 7 days after each immunization were titrated for agglutinins. The results are given in Tables 1, 2 and 3. Hormonal bursectomy affected anti-SRBC antibody responses only slightly with regard to both the rate and extent of the response. It is clear that the primary responses of TP-treated chickens to SRBC are similar to those of normal chickens except for the results of experiments 3 and 4. In contrast, antibody responses against the two bacterial antigens were suppressed so that most chickens failed to produce detectable agglutinins in the primary response. Secondary responses were also suppressed in some chickens. No BA-specific agglutinins were detected in any of the TP-treated chickens immunized at 3·5 and 5·5 weeks of age. Most antibodies produced by TP3 chickens in the

Table 3. Immune responses to *Salmonella pullorum* in testosterone–proprionate-treated chickens

Expt no.	Groups	Primary response				Secondary response			
		No treatment*		2-ME treatment†		No treatment		2-ME treatment	
		Responders‡	Titre§	Responders	Titre	Responders	Titre	Responders	Titre
Immunizations at 3·5 and 5·5 weeks of age									
1	TP3-H	0/5		0/5		2/4	2·6±2·1	0/4	
	0TP3-H	0/4		0/4		3/3	0·5	0/3	
	Con-H	9/9	1·5±1·4	0/9		9/9	5·5±1·3	9/9	1·2±0·5
2	TP3-A	0/12		0/12		6/9	2·1±1·2	0/9	
	Con-A	5/5	1·6±0·9	0/5		5/5	4·0±0·8	4/5	1·5±0·7
Immunizations at 4 and 6 weeks of age									
3	TP3-H	1/6	1·0	0/6		4/5	5·5±0·3	0/5	
	0TP3-H	0/7		0/7		2/4	1·5±0·7	0/4	
	Con-H	4/4	5·5±0·6	2/4	1·0	4/4	7·7±0·6	3/4	2·8±1·4
4	TP3-A	1/8	2·5	0/8		2/7	2·5±0·9	0/7	
	0TP3-A	0/6		0/6		0/6		0/6	
	Con-A	3/3	3·7±0·8	1/3	1·0	3/3	5·7±0·4	2/3	2·3±0·3
Immunizations at 6 and 9 weeks of age									
5	TP3-H	0/5		0/5		2/5	4·5±1·1	1/5	1·0
	0TP3-H	0/3		0/3		0/3		0/3	
	7TP3-H	0/3		0/3		1/3	1·0	0/3	
	Con-H	5/6	5·2±1·3	2/5	1·0	5/5	7·8±1·2	5/5	2·5±0·9
6	TP3-H	1/6	1·0	0/6		4/6	2·4±0·4	1/6	1·0
	0TP3-H	0/5		0/5		0/4		0/4	
	7TP3-H	0/5		0/5		0/3		0/3	
	Con-H	5/5	4·5±0·3	1/5	1·0	5/5	6·5±0·3	5/5	1·8±0·4

Control and TP-treated chickens were immunized intravenously at indicated ages with a mixture of SRBC, BA and SP antigens. Agglutinins were determined in sera taken a week after both the first and second stimulations.

* Titres in sera not treated with 2-ME.

† Titres in sera treated with 2-ME.

‡ Number of responding chickens per number of total chickens.

§ Mean titres of responders and standard deviation.

¶ Each antigen was injected into individual chickens.

primary response were 2-ME-sensitive. After the second immunization, however, some IgG appeared, although IgG responses against bacterial antigens were very low in comparison with that against SRBC.

Removal of the bursal remnants remaining in TP-treated neonatal chickens resulted in marked suppression of subsequent antibody responses. Presumably therefore, most immune responses of TP-treated chickens are dependent upon bursal remnants. The same profile of immune responses as that seen in TP3 chickens was found also in those bursectomized hormonally on the 10th or 12th day of embryonation (Hirota and Bito, 1975).

Relationship between the mode of immune response and morphological changes occurring in bursal remnants in individual TP-treated chickens

The bursal remnants were taken from 6·5-week-old TP3 chickens immunized with the mixture of the antigens at 3·5 and 5·5 weeks of age (experiments 1 and 2); eight to thirteen HE-stained sections prepared from each remnant. The total follicles in eight to thirteen sections were counted. Table 4 gives the mean number of follicles per section of the bursal remnant. The weight of bursal remnant and the mean number of follicles per section ranged from 4 to 513 mg and from 0 to 118, respectively. The two figures were virtually proportional to each other. Bursal follicles from TP3 chickens were denuded of cells and smaller in size than those from normal ones (Figs 1–5). A diffuse infiltration of lymphocytes, was observed in most sections from TP3 chickens (Fig. 5). Abnormal follicles occurred which lacked the boundary between inner and outer regions (Fig. 3) or were partially replaced by epithelial cells (Fig. 4). Abnormal follicles were considerably more abundant in TP3 chickens than in the normal ones.

Table 4. Immune responses to sheep red blood cells, *Brucella abortus* and *Salmonella pullorum* in individual chickens treated with testosterone–propionate

Chickens	SRBC response				BA response				SP response			
	Primary		Secondary		Primary		Secondary		Primary		Secondary	
	No*	ME†	No	ME	No	ME	No	ME	No	ME	No	ME
TP3-H-1	4	<1	8	<1	<1	<1	<1	<1	<1	<1	<1	<1
TP3-H-2	0·5	<1	8	<1	<1	<1	<1	<1	<1	<1	1	<1
TP3-H-3	2·5	<1	7	3	5·5	<1	5	2	<1	<1	4	<1
TP3-H-4	4·5	<1	6·5	4	<1	<1	<1	<1	<1	<1	<1	<1
TP3-A-1	<1	<1	5	<1	<1	<1	<1	<1	<1	<1	<1	<1
TP3-A-2	4	<1	5·5	3	<1	<1	<1	<1	<1	<1	2	<1
TP3-A-3	2	<1	6	1	<1	<1	<1	<1	<1	<1	2	<1
TP3-A-12	1·5	<1	6·5	<1	<1	<1	<1	<1	<1	<1	2·5	<1
TP3-A-13	1	<1	5	<1	<1	<1	<1	<1	<1	<1	<1	<1
TP3-A-14	3·5	<1	6	1·5	<1	<1	<1	<1	<1	<1	4	<1
TP3-A-15	4	<1	8	1	<1	<1	<1	<1	<1	<1	0·5	<1
TP3-A-17	2	<1	5	1	<1	<1	<1	<1	<1	<1	1·5	<1
TP3-A-18	2	<1	7	1	<1	<1	<1	<1	<1	1	2·5	<1
Con-H-1	2·5	<1	8	4	6	0·5	6	3	3	<1	7·5	0·5
Con-H-2	3·5	<1	8	3	5·5	1	5	1	1	<1	5	2
Con-H-3	2·5	<1	3	1	5·5	2	6·5	1	1	<1	6	1·5
Con-H-4	2	<1	6	4	6	2·5	5	3·5	1	<1	3·5	0·5
Con-H-5	4	<1	6	3	6	2	4	3	2·5	<1	4	1
Con-A-1	2·5	<1	5	3·5	3	1	3·5	2	1	<1	3	<1
Con-A-2	2	<1	4·5	<1	4	1	4	1	1	<1	3·5	1·5
Con-A-3	2	<1	6	4·5	4	2	5·5	2·5	1	<1	4	1·5
Con-A-4	3·5	<1	6	2·5	2·5	<1	5	2·5	3	<1	5	2
Con-A-5	3·5	<1	6·5	3	2	<1	4	1	2	<1	4·5	1

Antigenic challenges were made intravenously at 3·5 and 5·5 weeks of age with a mixture of SRBC, BA and SP antigens.

* Titres in sera not treated with 2-ME.

† Titres in sera treated with 2-ME.

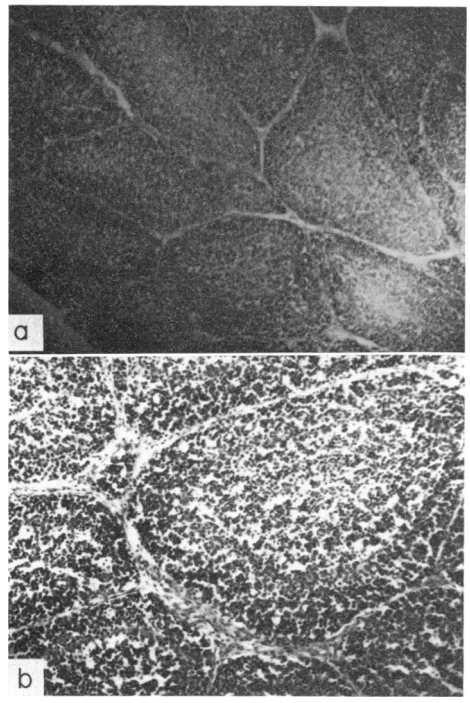

Figure 1. Bursa of a normal 6·5-week-old chicken. (H & E; magnification × 80.)

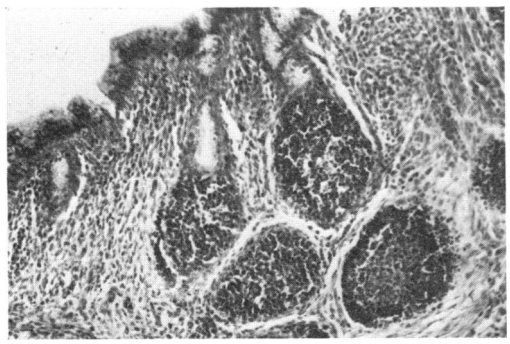

Figure 2. Bursa of a 6·5-week-old chicken treated with testosterone–propionate on the 3rd day of embryonation. The bursal follicles are atrophic and small. (H & E; magnification × 80.)

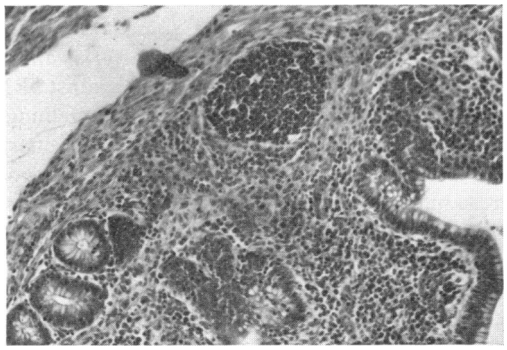

Figure 3. An abnormal follicle in the bursal remnant of a 6·5-week-old chicken treated with testosterone–propionate on the 3rd day of embryonation. No boundary between cortical and medullary regions is found. (H & E; magnification × 80.)

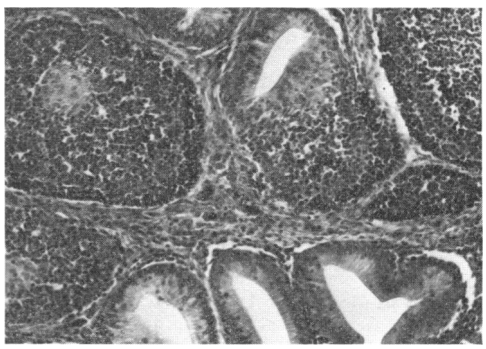

Figure 4. A degenerating follicle with partial epithelialization in the bursal remnant of a 6·5-week-old chicken treated with testosterone–propionate on the 3rd day of embryonation. (H & E; magnification × 80.)

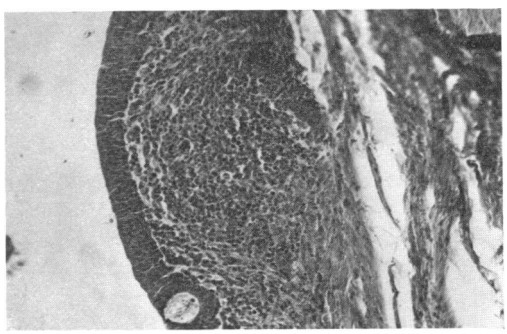

Figure 5. Diffuse lymphocyte infiltration in the bursal remnant of a 6·5-week-old chicken treated with testosterone–propionate on the 3rd day of embryonation. (H & E; magnification × 80.)

Table 5. Histological findings of bursal remnants in individual chickens treated on the 3rd day of egg incubation with testosterone–propionate

Chickens	Wet weight of remnants (mg)	Number of complete follicles	Features
TP3-H-1	11	0	Lymphocytic infiltration
TP3-H-2	4	3	Significant organization
TP3-H-3	513	118	Almost normal finding
TP3-H-4	4	8	Epithelialization of follicles
TP3-A-1	11	0	Lymphocytes are very few in number; significant organization
TP3-A-2	425	114	Epithelialization of follicles
TP3-A-3	44	25	Epithelialization of follicles
TP3-A-12	22	0	Lymphocytes are very few in number; organization; epithelialization of follicles
TP3-A-13	161	17	Epithelialization of follicles; organization
TP3-A-14	110	58	Epithelialization of follicles
TP3-A-15	37	11	Epithelialization of follicles
TP3-A-17	92	32	Epithelialization of follicles
TP3-A-18	51	13	Epithelialization of follicles
Con-H	1890±685*	487±125	

Autopsies were done at 6·5 weeks of age (see Materials and Methods section).

* Mean weight and standard deviation.

† Mean number of complete follicles and standard deviation.

The production of total antibody to SRBC correlated very slightly with the presence of bursal follicles (Tables 1, 4 and 5). Such TP3 chickens as TP3-A-1 and TP3-A-12 with bursal remnants containing only a small number of lymphocytes exhibited extremely low primary anti-SRBC response. This suggests that the anti-SRBC response may be dependent upon descendants of bursal lymphoid cells even if it is independent of the presence of bursal follicles. TP-treatment blocked almost completely the immune response against BA antigen (Tables 2 and 4).

Most TP3 chickens gave secondary responses against SP antigen, although primary responses were completely prevented (Tables 3 and 4). The presence of bursal follicles correlated well with the primary immune response against the bacterial antigens in comparison of TP3 and control chicken groups (Tables 4 and 5). In individual TP3 chickens, the relationship was not upheld. One chicken (TP3-H-3) responded against BA and SP antigens with a pattern similar to normal after the second immunization. This chicken possessed a large remnant containing 118 follicles per section. The remnant was similar to the normal one in the density of the follicles in the tissue. TP treatment strongly suppressed the IgG antibody response and the development of bursal follicles. TP3 chickens with remnants containing a relatively large number of follicles produced IgG antibody, for example such chickens as TP3-H-3 and TP3-A-2. Chickens such as TP3-A-3, TP3-A-15, TP3-A-17 and TP3-A-18 which possessed a small number of bursal follicles responded with the production of IgG anti-SRBC antibody to low titres. Thus, there was a correlation between the existence of follicles and IgG antibody production.

DISCUSSION

The present results can be summarized as follows: (a) production of SRBC-specific IgM antibody was only slightly affected by TP treatment performed during embryonation; it may not depend therefore upon the presence of bursal follicles, (b) TP treatment early in embryonic life strongly suppressed primary immune responses against bacterial antigens. The presence of follicles correlated with the primary antibody responses to the two bacterial antigens used. The number of bursal stem cells with potential for immunocompetence may be reflected by the

magnitude of the primary response rather than the secondary response, which is dependent largely upon cell proliferation, (c) TP treatment strongly suppressed IgG antibody production. The production of IgG antibody correlated with presence of bursal follicles.

Lymphoblasts first appear in primitive bursal follicles on the 14th or 15th day of embryonation. The lymphoblasts gradually differentiate into large, medium and small lymphocytes. Small lymphocytes appear in bursal follicles of 16–18-day-old embryos. Lymphocytes appear also in the outer region of follicles during this period. The medullary and cortical regions separated by the basal membrane are observed in 17–18-day-old embryos (Ackerman and Knouff, 1959, 1964; Kobayashi, 1968). The bursa completes development at 8 days of age (Sato, 1971). Bursal lymphocytes may undergo differentiation and maturation through these processes including follicle formation. Thus mature bursal lymphoid stem cells acquire the potential to respond against bacterial antigens as well as SRBC and switch from IgM to IgG antibody production in immunization after subsequent peripheralization. Another explanation for the change from IgM to IgG response is that the IgG-producing cell clone may arise from the IgM-producing one in the bursa without contact with the antigen (Kincade, Lawton, Bockman and Cooper, 1970). Treatment of the chick embryo with TP suppresses the development of bursal follicles completely or incompletely, possibly by blocking the development of follicles. TP seems to affect the lymphocytes only slightly. Lymphocytes affected by TP could migrate to the peripheral lymphoid organs without the development of follicles and further intrabursal maturation. Most of them may be capable of producing antibodies against SRBC but not bacterial antigens. They may be able to respond with IgM but not with IgG antibody production.

Surgical extirpation of bursal remnants did not always annul antibody responses (Tables 1, 2 and 3), possibly since the peripheralization of immunocompetent cells from the TP-affected bursa occurs during embryonic life. Possibly also the cells mature in 'non-bursal sites' (Lerner *et al.*, 1971; Bryant, Adler, Cordy, Shifrine and DaMassa, 1973; Sato, 1973). Bryant and his colleagues (1973) found that follicles were absent from the bursa of TP-treated chickens at 10 weeks of age, at which time bursal involution should have already proceeded to a considerable extent from our observation. Histological examinations at younger ages are obviously needed. Experiments using adoptive immunization to determine whether non-bursal maturation sites exist are in progress in this laboratory.

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