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The B Cell Development Independent of the Bursa of Fabricius but Dependent upon the Thymus in Chickens Treated with Testosterone-propionate

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Abstract

Most chickens treated with testosterone-propionate on the 12th day of egg incubation (TP12 chickens) and immunized with a mixture of sheep red blood cells, *Brucella abortus* and *Salmonella pullorum* produced exclusively IgM antibodies against Sheep red blood cells without the production of IgG antibodies against sheep red blood cells and of antibodies against the bacterial antigens. The development of the bursa in the TP12 chickens was suppressed extremely, and the bursae hardly possessed follicle structures. Spleen cells taken from chickens treated with testosterone-propionate, surgically bursectomized and immunized were treated with anti-bursa cell antiserum in the presence of complement, and toghther with the mixed antigens were transferred into immunodeficient recipient chickens. The recipient chickens restored the capacity to elicit the production of antibodies against sheep red blood cells. In contrast, adoptive immune responses by spleen cells from normal chickens were obliterated by treating the cells with anti-bursa cell antiserum in the presence of complement. The TP12 chickens were thymectomized at 7 days of age (TP12–ST7 chickens) and were immunized with the mixed antigens. The chickens thus treated were totally immunoincompetent. Thymocytes and thymus derived cells taken from normal chickens were introduced into the TP12–ST7 chickens, but the chickens remained totally immunoincompetent. Spleen cells taken from the TP12–ST7 chickens together with spleen cells, containing T cells but not B cells, from normal chickens and with the mixed antigens were injected into immunodeficient recipient chickens. The recipient chickens did not produce antibodies at all indicating the absence of immunocompetent B cells in the spleen of the TP12–ST7 chicken. These findings are discussed in relation to an unusual type of the B cell development in the chicken treated with testosterone-propionate.

Introduction

Hirota, Suzuki, Chazono and Bito[1] found that chickens treated with testosterone-propionate (TP) in embryonic life expressed to develop exclusively the capacity to produce IgM antibodies against sheep red blood cells (SRBC) in striking contrast to the substantial lack of the capacity to produce IgG antibodies against SRBC and antibodies against *Brucella abortus* (BA) and *Salmonella pullorum* (SP), when the chickens were immunized with a mixture of the three antigens. The capacity to produce IgM anti-SRBC antibodies was developed even in the environment in which the bursal structure, especially its follicle structure was completely obliterated with the TP-treatment. In striking contrast, the capacities to produce IgG antibodies against SRBC and antibodies against BA and SP were developed depending strictly upon the integral structure of a

bursal follicle. The authors noticed with great interest such diverse influences of the TP-treatment in embryonic life on the development of various antigen– and immunoglobulin-class-specific immunocompetences.

Thus, we attempted to clarify the mode of development of the immunocompetence which is resistant to the TP-treatment in embryonic life and is not dependent upon the integrity of the bursal follicle. We attempted to examine with special interest whether functional B cells developed in the bursal environment extremely damaged by the TP-treatment may possess bursa cell-specific cell surface antigen (s), which normal B cells possess necessarily, or not, and whether thymus-dependence of anti-SRBC immune responses by the TP-treated chickens may be derived from the necessity of T cell help for anti-SRBC immune responses or from the dependence of B cell development upon the thymus. The present experiments suggest strongly that the development of functional B cells in the TP-treated chickens is almost independent of the bursa but rather dependent upon the thymus.

Materials and Methods

Chickens

Chickens used were White Leghorn inbred strain Anthony from our own flocks. Strain Anthony possesses B^{11}– and B^{13}–genes, and is of genotype $B^{11}B^{11}$, $B^{13}B^{13}$ and $B^{11}B^{13}$. Individuals within $B^{11}B^{11}$– and $B^{13}B^{13}$–genotypes are homozygous, and those within $B^{11}B^{13}$–genotype heterozygous, at the major histocompatibility B locus. Chickens within $B^{11}B^{11}$–, $B^{13}B^{13}$– and $B^{11}B^{13}$–genotypes were used as donors, and chickens within $B^{11}B^{13}$–genotype as recipients, exclusively. It was confirmed by the following experiments that donor and recipient chickens are histocompatible. (a) 1×10^7 peripheral blood leucocytes of donor chickens were inoculated on the chorioallantoic membrance of 13-day embryos of recipient chickens. No foci were produced on the membrane indicating the negative graft-versus-host reaction. The negative graftversus-host reaction was comfirmed also by the lack of splenic swelling in the recipient chickens. (b) A piece of the skin taken from the donor chicken was placed and sewn up in the direction turned the other way round in the area from where the skin had been removed on the body surface of the recipient chicken. The opposite direction of the feather allowed easy location of the implant. The implants of donor chickens settled firmly on the body surface of the recipient chickens throughout the observation period of 63 days or more. In contrast, the implants falled off 7 to 9 days after the transplantation in the histoincompatible combination of donor and recipient chickens. (c) It has been always observed in a number of cell transfer experiments that the introduction of immunologically normal lymphoid cells of the donor chickens into the immunodeficient recipient chickens restored successfully the immunoincompetence of the recipient chickens.

Treatment of chickens

TP was purchased from Sigma Chemical Company, St Louis, Missouri. Three– and 12-day-old embryonated eggs were terated with TP by the two following methods; TP (4 mg) dissolved in 0.1 ml of corn oil was injected into chorioallantoic fluid of 12-day-old embryonated eggs. Chickens thus treated were designated as TP12 chickens. Three-day-old embryonated eggs were dipped into 2% TP solution in ethanol for 5 seconds. The

chickens were designated as TP3 chickens. Bursal remnants were surgically extirpated at hatching from some of TP12 and TP3 chickens. These chickens thus treated were designated as 0TP12 and 0TP3 chickens, respectively. Untreated chickens (Con chickens) were used as the control. Some of TP12, 0TP12 and control chickens were thymectomized surgically at the age of 7 days (TP12–ST7, 0TP12–ST7 and Con–ST7 chickens, respectively). Some of 0TP3 chickens were thymectomized surgically on the day of hatching (0TP3–ST0 chickens). The physical state and mortality of chickens treated with TP and thymectomized were not different from those of chickens treated with TP but not thymectomized. A small number of TP-treated chickens were weakened at the age of 8 weeks or older, and some of them died. The weakened chickens were omitted from experiments as a matter of cource. The physical state of chickens which were treated with TP and survived and of chickens which were treated with TP, were thymectomized and survived was not different from that of normal chickens.

Antigens

The antigens used were SRBC, BA and SP strain 4054. SRBC was 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[2], inactivated by treating with 0.1% formalin in phosphate-buffered saline (PBS) and washed twice with PBS.

Immunization

A mixture of 1×10^8 SRBC and 5×10^8 each of BA and SP cells was injected intravenously into each chicken at the age of days indicated in each experiment.

Antibody titration

Antisera were taken from chickens immunized one week after immunization. Agglutinins to SRBC were measured by microtitration with 25 μl volumes of the serum to be titrated in two-fold dilutions and of 0.5% SRBC (wet weight/volume). PBC was used as diluent. The plates were incubated at 37° for 2 h and at 4° overnight. Agglutinins to BA and SP antigens were titrated by a technique similar to that used for determination of anti-SRBC agglutinins except that 0.2% suspensions of BA and SP cells (wet weight/volume) were 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 $\log_2$. The number of responders in each group and mean ± s.d. $\log_2$ titre of responders are given in the tables.

Antibody titration after treatment with 2-mercaptoethanol (2-ME)

In our preliminary experiments, the treatment with 2–ME of antisera showed that the antibody activities of macroglobulin fractionated by salting out with ammonium sulfate and gel filtration on a Sephadex G–200 column were sensitive to reduction by 2–ME. The serum was added to an equal volume of 0.2M 2–Me; the mixture was allowed to stand for 40 minutes 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 and SP.

Microscopic examination

Seven days after the last immunization with a mixture of SRBC, BA and SP bursal remnants were taken from TP-treated and control chickens and fixed with 10% formalin. Eight to thirteen sections were prepared from each remnant, and stained with haematoxylin and eosin.

Adoptive immunization

Cell suspensions were prepared from spleenss of donors in the following procedures. Spleens were dissected into cold medium 199; the tissues were cut into pieces with scissors. The suspensions were filtered through layers of gauze after agitation with a pipette to break up the cell aggregates. The cells were washed three times by centrifugation at 200 g for 10 minutes. The viability of the cells was examined by trypan blue dye exclusion. Donor cells were injected intravenously into immunologically inactive recipient chickens together with a mixture of SRBC, BA and SP. Details of donor and recipient chickens will be described in the text. Some of chickens to be used as a recipient chicken were subjected to X-ray irradiation under the following condition; the chickens received 500R at a dose rate of 50R per minute at a distance of 55cm. The X-ray irradiation unit was run at 200KV, 20mA and with a 0.3mm Cu– and 0.5mm Al-filter.

Preparation of anti-bursa cell antiserum and treatment of donor cells with the antiserum

Rabbits were subcutaneously injected with 3×10^8 bursa cells taken from 2-week-old chickens together with Freund's complete adjuvant. Several booster injections with the same number of mixed bursa cells frm 3– to 8-week-old chickens were intravenously given a month later at 2-week intervals. The rabbits were bled 10 days after the last immunization, and the sera were inactivated by heating at 56° for 30 minutes. One millilitre of pooled antiserum diluted 4-fold with PBS was absorbed at room temperature for one hour first with 0.1 ml of packed chicken erythrocytes. Furthermore, 1 ml of the antiserum was absorbed three times with 2.5×10^8 thymus cells from 2–, 7– and 8-week-old chickens deprived of the B lymphoid system by surgical bursectomy and X-ray irradiation at hatching by allowing each mixture to stand at room temperature for one hour and at 4° overnight. One millilitre of thus absorbed antiserum was again absorbed at room temperature for 60 minutes with 17 mg of chicken immunoglobulin until the serum became nonagglutinable with SRBC coated with chicken immunoglobulin. We called anti-bursa cell antiserum thus absorbed with the three factors 'ABuS'. Packed cells (5×10^7) to be examined in adoptive immunization were suspended in 0.5 ml of ABuS diluted 32-fold with PBS and 0.5 ml of complement (a fresh pooled serum of guinea pigs) diluted 4-fold with PBS. The suspension was incubated at 37° for 45 minutes, and examined for viability by the trypan blue dye exclusion test after two washings with PBS. ABuS diluted 32-fold exhibited the cytotoxicity of 100% to bursa cells from 7-day-old chickens under co-operation with complement and was innocuous to thymus cells from the chickens of the same age.

Preparation of anti-thymus cell antiserum and treatment of cells with the antiserum

Thymus cells were taken from 2-week-old chickens surgically bursectomized at hatching. A first dose of the 2×10^8 thymus cells together with Freund's complete

adjuvant was injected into the four footpads of rabbits, and then three injections with the same dose of thmus cells were intravenously given one week later at weekly intervals. The rabbits were bled 7 days after the last immunization, and the sera were pooled and were inactivated by heating at 56° for 30 minutes. One millilitre of the antiserum diluted 4-fold with PBS was absorbed at room temperature for one hour first with 0.1 ml of packed chicken erythrocytes, and then three times with 2.5×10^8 bursa cells from 2-week-old chickens by allowing the mixture to stand at room temperature for one hour and at 4° overnight. The treatment of cells with the anti-thymus cell antiserum thus obtained was carried out by the same procedures with the by ABuS. The antiserum diluted 32-fold exhibited cytotoxicity of 100% to thymus cells from 7-day-old chickens and was innocuous to bursa cells from chickens of the same age.

Results

Antibody responses by chickens treated with testosterone propionate and morphological finding of their bursal remnants

Chickens of strain Anthony were treated with 4 mg of TP on the 12th day of egg incubation and were intravenously immunized at 4, 6 and 8 weeks of age with a mixture of SRBC, BA and SP. The sera taken a week after each immunization were titrated for agglutinins. The TP12 chickens produced no detectable antibodies to the three antigens one week after primary immunization. Most TP12 chickens produced exclusively IgM anti-SRBC agglutinins in the secondary and tertiary responses, and only a small number of TP12 chickens produced IgG anti-SRBC antibodies to low titres in addition to IgM antibodes (Table 1). In contrast, agglutinins against the bacterial antigens were hardly

Table 1. Antibody responses by chickens treated with testosterone-propionate in embryonic life and immunized with a mixture of sheep red blood cells, *Brucella abortus* and *Salmonella pullorum* at 4, 6 and 8 weeks of age (Expt no. 1) or at 4, 6 and 10 weeks of age (Expt no. 2)

Expt no.	Groups	Anti-gens	Secondary response				Tertiary response			
			No treatment*		2-ME treatment†		No treatment		2-ME treatment	
			Respond-ers	Titres$^{\xi}$	Respond-ers	Titres	Respond-ers	Titres	Respond-ers	Titres
1	TP12	SRBC	26/38	4.6 ±2.7	4/38	1.4 ±0.4	36/38	5.0 ±3.0	10/38	2.5 ±0.9
		BA	0/38		0/38		3/38	1.8 ±0.5	0/38	
		SP	0/38		0/38		3/38	2.6 ±1.1	0/38	
	0TP12	SRBC	1/10	1.0	0/10		5/8	1.2 ±0.3	0/8	
		BA	0/10		0/10		0/10		0/10	
		SB	0/10		0/10		0/10		0/10	
	Con	SRBC	9/9	6.1 ±0.8	9/9	2.6 ±0.9	9/9	7.4 ±0.9	9/9	4.7 ±1.9
		BA	9/9	6.7 ±0.8	9/9	3.1 ±0.8	9/9	5.6 ±0.8	9/9	3.0 ±0.7
		SP	9/9	6.1 ±1.2	9/9	3.6 ±1.2	9/9	6.6 ±1.1	9/9	2.7 ±0.7
2	0TP3	SRBC	11/16	4.5 ±2.1	0/16		3/6	7.0 ±1.0	0/6	
		BA	0/16		0/16		1/6	1.0	0/6	
		SP	0/16		0/16		0/6		0/6	
	Con	SRBC	8/8	5.8 ±0.6	8/8	3.8 ±1.2	5/5	8.3 ±2.3	5/5	4.5 ±0.8
		BA	8/8	6.1 ±2.6	8/8	2.3 ±0.7	5/5	7.5 ±0.4	5/5	3.8 ±0.5
		SP	8/8	4.8 ±0.6	8/8	1.9 ±0.5	5/5	7.8 ±1.2	5/5	2.5 ±0.9

* Titres in sera not treated with 2-ME.
† Titres in sera treated with 2-ME.
Φ Number of responding chickens per number of total chickens in a group.
ξ Mean $\log_2$ titres of responders and standard deviation.

produced by the immunized TP12 chickens. Surgical extirpation of bursal remnants on the day of hatching deprived the TP12 chickens considerably of primary and secondary responses. However, TP12 chickens thus treated (0TP12 chickens) exhibited a slight IgM antibody response after the third immunization. Bursal remnants of the TP12 chickens were examined morphologically. The sizes of the bursal remnants ranged from so small as invisible with the naked eye to about 1/8 the normal bursal volume, the average being about 1/100 the normal bursal volume, and the mean number of bursal follicles per stained section ranged from 0 to 2.3 in striking contrast to 500 or more in the normal bursa. Major portion of a bursal remnant was replaced by pronounced fibrosis and residual follicles were denuded of cells. Another group of chickens (strain Anthony) were treated with TP on the third day of egg incubation, surgically bursectomized and immunized with the mixed antigens at 4, 6 and 10 weeks of age followed by antibody titration a week after each immunization. Half of the 0TP3 chickens produced IgM anti-SRBC antibodies of a level comparable to that of the responses by normal chickens immunized in striking contrast to no detectable level of IgG anti-SRBC response. The 0TP3 chickens hardly produced antibodies against the bacterial antigens.

Bursal remnants of the TP3 chickens were examined morphologically. The degre of morphological abnormality observed in the bursal remnants of the TP3 chickens was slightly lower than that of the TP12 chickens in both the bursal volume and the number of bursal follicles.

Adoptive antibody responses by spleen cells taken from testosterone-propionate-treated chickens and treated with antibursa anti-bursa cell antiserum

In order to further elucidate the degree of the bursa-de-pendence of B cell development within environment of the bursa strongly suppressed by the TP-treatment, the authors attempted experiments in which spleen cells taken from the TP-treated chickens and treated with anti-bursa cell antiserum in the presence of complement are examined for the immunocompetence using a *in vivo* culture technique. The cytotoxicity test with the use of ABuS showed that spleen cells from normal chickens aged 7, 9 and 11 weeks contained 32%, 33% and 36%, respectively, of bursa-derived cells. A highly specific antiserum directed toward bursa cells was prepared from rabbits as described in the section of Materials and Methods. Spleen cells from 11-week-old 0TP3 chickens immunized with the mixed antigens at 4, 6 and 10 weeks of age, in an experiment described as 'Expt no. 2' in Table 1, were used as donor cells to be examined. Chickens to be used as a recipient chicken were surgically bursectomized at hatching, injected intraperitoneally with 2.5 mg of cyclophosphamide for 2 consecutive days starting on the day of hatching and received 500 R of X-ray irradiation at 6 days of age. Chickens thus treated were designated as SB0-CYX chickens. The 2×10^7 spleen cells from 0TP3 chickens immunized were treated with ABuS in the presence of complement and were transferred into the SB0-CYX chickens together with the mixed antigens. The recipient chickens received an additional immunization a week later, and agglutinins in sera taken a week after each immunization were titrated. Five out of six recipients injected with the ABuS-treated spleen cells and the antigens produced IgM anti-SRBC agglutinins of titres near to those produced by untreated spleen cells from the 0TP3 chickens immunized and to those produced by untreated spleen cells from normal chickens immunized (Table 2). In contrast, spleen cells taken from normal chickens similarly immunized and treated with ABuS in the presence of complement could not elicit the antibody response at all. This finding indicates clearly that antibody-forming cells developed in 0TP3 chickens do

Table 2. Adoptive antibody responses by spleen cells taken from TP-treated chickens and treated with anti-bursa cell antiserum in the presence of complement.

Cells transferred (2×10^7)	Treatment with ABuS	Antigens	Primary response		Secondary response	
			Responders	Titres	Responders	Titres
0TP3–spleen cells*	–	SRBC	4/4	3.8 ±0.8	4/4	5.5 ±1.4
		BA	0/4		0/4	
		SP	0/4		0/4	
0TP3–spleen cells	+	SRBC	3/6	3.2 ±1.2	5/6	4.8 ±1.5
		BA	0/6		0/6	
		SP	0/6		0/6	
Con–spleen cells	–	SRBC	4/5	4.8 ±1.6	5/5	5.2 ±1.6
		BA	5/5	2.5 ±0.8	5/5	3.9 ±1.2
		SP	5/5	5.2 ±1.2	5/5	4.6 ±2.4
Con–Spleent cells†	+	SRBC	0/5		0/5	
		BA	0/5		0/5	
		SP	0/5		0/5	
——	–	SRBC	0/3		0/3	
		BA	0/3		0/3	
		SP	0/3		0/3	

* Spleen cells from 11-week-old chickens treated with TP on the third day of egg incubation, surgically bursectomized on the day of hatching and immunized with the mixed antigens at 4, 6 and 10 weeks of age.

† Spleen cells from 11-week-old normal chickens immunized with the mixed antigens at 4, 6 and 10 weeks of age.

The other abbreviations are the same with those in Table 1.

not possess cell surface antigen (s) specific for normal B cells developed in the integral bursal environment although normal antibody-forming cells possess the antigen (s) as a matter of course.

Antibody responses by chickens treated with TP and thymectomized

To determine whether the development of the capacity to respond to SRBC with IgM antibody production in the TP-treated chickens are thymus-dependent or not, TP12 chickens were thymectomized surgically at 7 days of age, immunized with the mixed antigens at 4, 6 and 8 weeks of age and measured for antibody production. No agglutinin was detected at all (Table 3). Chickens not treated with TP but surgically thymectomized 7 days after hatching exhibited antibody responses of slightly lower levels than those by untreated control chickens immunized. The 0TP3 chickens were thymectomized at hatching and immunized with the same antigens at 4, 6 and 10 weeks of age. Chickens thus treated were virtually immunoincompetnet.

Antibody responses by chickens treated with testosterone-propionate, thymectomized and transplanted with thymocytes and thymus-derived cells

In order to restore the immunologically inactive TP12–ST7 and 0TP12–ST7 chickens, the transfer of thymocytes and thymus-derived cells into these chickens was attempted. The cytotoxicity test with the use of anti-thymus cell antiserum showed that

Table 3. Antibody responses by chickens treated with testosterone-propionate in embryonic life, neonatally thymectomized and immunized with a mixture of sheep red blood cells, *Brucella abortus* and *Salmonella pullorum* at 4, 6 and 8 weeks of age (Expt no. 1) or 4, 6 and 10 weeks of age (Expt no. 2)

Expt no	Groups	Antigens	Secondary response				Tertiary response			
			No treatment		2-ME treatment		No treatment		2-ME treatment	
			Responders	Titres	Responders	Titres	Responders	Titres	Responders	Titres
1	TP12-ST7	SRBC	0/5		0/5		0/5		0/5	
		BA	0/5		0/5		0/5		0/5	
		SP	0/5		0/5		0/5		0/5	
	0TP12-ST7	SRBC	0/6		0/6		0/6		0/6	
		BA	0/6		0/6		0/6		0/6	
		SP	0/6		0/6		0/6		0/6	
	Con-ST7	SRBC	10/11	5.0 ±1.0	10/11	1.8 ±0.5		NT*		
		BA	9/11	5.9 ±1.6	5/11	1.8 ±1.2		NT		
		SP	10/11	4.6 ±1.2	6/11	1.8 ±1.4		NT		
	Con	SRBC	9/9	6.1 ±0.8	9/9	2.6 ±0.9	9/9	7.4 ±0.9	9/9	4.7 ±1.9
		BA	9/9	6.7 ±0.8	9/9	3.1 ±0.8	9/9	5.6 ±0.8	9/9	3.0 ±0.7
		SP	9/9	6.1 ±1.2	9/9	3.6 ±1.2	9/9	6.6 ±1.1	9/9	2.7 ±0.7
2	0TP3-ST0	SRBC	1/6	1.0	0/6			NT		
		BA	0/6		0/6					
		SP	0/6		0/6			NT		
	Con	SRBC	8/8	5.8 ±0.6	8/8	3.8 ±1.2	5/5	8.3 ±2.3	5/5	4.5 ±0.8
		BA	8/8	6.1 ±2.6	8/8	2.3 ±0.7	5/5	7.5 ±0.4	5/5	3.8 ±0.5
		SP	8/8	4.8 ±0.6	8/8	1.9 ±0.5	5/5	7.8 ±1.2	5/5	2.5 ±0.9

* Not tested.
The other abbreviation are the same with those in Table 1.

spleen cells from normal chickens aged 9 weeks contained 42% of thymus-derived cells. The TP12–ST7 and 0TP12–ST7 chickens aged one week received an intravenous injection of 10^8 thymocytes taken from 1-week-old normal chickens, were immunized with the mixed antigens at 4, 5 and 6 weeks of age, and were further injected at 7 weeks of age with 10^8 ABuS-treated spleen cells (as a source of thymus-derived cells or T cells) from 7-week-old chickens together with the antigens followed by an additional immunization after a week. Agglutinins in sera taken one week after each immunization were titrated. The chickens remained totally immunoincompetent (Table 4). The result was the same, also when thymocytes and ABuS-treated spleen cells, respectively, from 5.5– and 7-week-old chickens were implanted into the TP12–ST7 chickens during repeated immunizations.

Adoptive antibody responses by spleen cells from chickens treated with testosterone-propionate and thymectomized

Since the above experiments showed that transplantation of the TP12–ST7 and 0TP12–ST7 chickens with thymocytes and thymusderived cells did not restore their entirely deficient capacity to produce antibodies, spleen cells from the TP12–ST7 chickens were examined for the immunocompetence. Spleen cells from 8-week old TP12–ST7 chickens together with ABuS-treated spleen cells from normal chickens (as a

Table 4. Effects of transferring thymocytes and ABuS-treated spleen cells on antibody responses against sheep red blood cells by chickens treated with testosterone-propionate and surgically thymectomized.

Groups	Cells transferred (1×10^8 cells)		Response after transfer of thymocytes (tertiary response)				Response after additional transfer of ABuS-treated spleen cells (the 5th response)			
	Thymocytes (age of donor)	Spleen cells treated with ABuS	No treatment		2-ME treatment		No treatment		2-ME treatment	
			Responders	Titre	Responders	Titre	Responders	Titre	Responders	Titre
TP12-ST7	+ (1 wk)	+ (7 wks)	0/6		0/6		0/4		0/4	
0TP12-ST7	+ (1 wk)	+ (7 wks)	0/6		0/6		0/6		0/6	
Con-ST7	+ (1 wk)	+ (7 wks)	4/4	6.3 ±0.5	4/4	4.8 ±0.4	3/3	8.0	3/3	6.5 ±0.5
TP12-ST7	+ (5.5 wks)	+ (7 wks)	0/5		0/5		0/5		0/5	
0TP12-ST7	+ (5.5 wks)	+ (7 wks)	0/5		0/5		0/5		0/5	
Con-ST7	+ (5.5 wks)	+ (7 wks)	4/4	5.8 ±1.0	4/4	3.3 ±0.4	4/4	8.5 ±1.3	4/4	4.8 ±0.8
Con-ST7	–	–	4/4	5.8 ±1.0	4/4	2.0 ±1.0	4/4	7.5 ±1.5	4/4	3.5 ±1.0
Con	–	–	3/3	6.0	3/3	4.7 ±1.9	3/3	7.8 ±1.3	3/3	5.8 ±1.0

The TP12-ST7 and 0TP12-ST7 chickens aged one week received an intravenous injection of 10^8 thymocytes from one- or 5.5-week-old normal chickens, and were immunized intravenously with the mixed antigens at 4, 5 and 6 weeks of age. The thymocytes from 5.5-week-old donors were treated with ABuS in the presence of complement before transfer. Furthermore, they were intravenously injected at 7 weeks of age with 10^8 spleen cells (as a source of thymus-derived cells or T cells) which had been taken from 7-week-old normal chickens and had been treated with ABuS in the presence of complement, together with the mixed antigens (the 4th immunization), and received one additional immunization (the 5th immunization) one week later. Agglutinins in sera taken one week after each immunization were titrated. The other abbreviations are the same with those in Table 1.

Table 5. Adoptive antibody responses by spleen cells from chickens treated with testosterone-propionate and thymectomized under supplement with spleen cells treated with anti-bursa cell serum.

Cells transferred ($\times 10^7$)	Antigens	Primary response				Secondary response			
		No treatment		2-ME treatment		No treatment		2-ME treatment	
		Responders	Titre	Responders	Titre	Responders	Titre	Responders	Titre
TP12-ST7- spleen cells* (2.5)	SRBC	0/6		0/6		0/6		0/6	
	BA	0/6		0/6		0/6		0/6	
	SP	0/6		0/6		0/6		0/6	
TP12-ST7- spleen cells + T cells† (2.5 + 2.5)	SRBC	0/6		0/6		0/6		0/6	
	BA	0/6		0/6		0/6		0/6	
	SP	0/6		0/6		0/6		0/6	
Con-B cells Φ + T cells (2.5 + 2.5)	SRBC	3/5	2.5 ±0.8	0/5		3/4	3.0	1/4	1.0
	BA	4/5	2.5 ±0.4	0/5		4/4	4.2 ±0.4	1/4	1.0
	SP	4/5	2.5 ±0.4	0/5		4/4	3.0	1/4	1.0
——	SRBC	0/4		0/4		0/4		0/4	
	BA	0/4		0/4		0/4		0/4	
	SP	0/4		0/4		0/4		0/4	

* Spleen cells taken from 8-week-old chickens treated with TP on the 12th day of egg incubation and surgically thymectomized at 7 days of age.

† Spleen cells taken from 8-week-old normal chickens and treated with ABuS in the presence of complement.

Spleen cells taken from 8-week-old normal chickens and treated with ABuS in the presence of complement.

Φ Spleen cells taken from 8-week-old normal chickens and treated with ATS in the presence of complement.

The other abbreviations are the same with those in Table 1.

T cell source) and the mixed antigens were transferred into chickens from which the bursa and the thymus had been surgically removed in the newly hatched period, followed by an additional immunization after a week. Antibodies in sera taken 7 days after each immunization were titrated. The spleen cells from the TP12–ST7 chickens were immunologically inactive, irrespective of supplement with ABuS-treated spleen cells from normal chickens (Table 5). This finding indicates that the spleens from the TP12–ST7 chickens contained no functional B cells.

Discussion

The bursa of Fabricius of TP-treated chickens was extremely abnormal in the morphological finding. The sizes of their bursal remnants were 1/100 to 1/10 the volume of the normal bursa, and te numbers of bursal follicles were 1/200 to 1/20 those of normal chickens. Major portion of a bursal remnant was replaced by pronounced fibrosis and residual follicles were denuded of cells. Most of the TP-treated chickens bearing so severly deformed bursae were competent exclusively for IgM anti-SRBC immune responses, lacking in the competences for IgG anti-SRBC immune responses and for immune responses to the bacterial antigens. The neonatal removal of bursal remnants from the TP-treated chickens resulted in the considerable decrease in the IgM anti-SRBC antibody response by the chickens, but the chickens preserved the immunocompetence to some extent. The treatment of a chicken in which bursal growth is strongly suppressed by TP injection in embryonic life and in which furthermore a bursal remnant is removed surgically on the day of hatching is a procedure thoroughly obliterating bursal influences from the chicken. Experiments were designed to determine whether antibody-forming cells developed in such an abnormal environment of chickens treated with TP and bursectomized may bear cell surface antigen (s) specific for bursa cells, which normal B cells bear necessarily, or not. Spleen cells taken from 0TP3 chickens immunized with the mixed antigens were treated with anti-bursa cell antiserum in the presence of complement, and together with the mixed antigens were injected into immunologically impaired recipient chickens. The cells elicited adoptive IgM anti-SRBC antibody responses in the recipient chickens in striking contrast to the total lack of adoptive immune responses by control cells treated with ABuS in the presence of complement (Table 2). This datum indicates clearly that the antibody-forming cells developed in the 0TP3 chickens bear no cell surface antigen (s) specific for normal bursa cells. The result suggests the two following possibilities: (a) Chickens subjected to the TP-treatment followed by surgical bursectomy might generate B cells competent for restricted immune responses from a site other than the bursa. (b) Since stem cells might be educated in a markedly abnormal environment of the bursa extremely damaged by the TP-treatment and surgical bursectomy, the cells might not be aboe to acquire cell surface antigen (s) specific for that (those) of bursa cells, and might result in only an incomplete maturation characterized by a restricted and biassed immune responsibility. It remains to be elucidated which of the two possibilities is valid. Fitzsimmons, Garrod and Garnett[3] and Janković, Isaković, Marković, Rajcevic and Knezevic[4] proposed nonbursal origin of humoral immunity based upon experiments showing that chickens surgically bursectomized at very early periods of embryonic life had capacities to produce antibodies and plaque-forming cells. However, the chickens subjected to early embryonic surgical bursectomy produced IgG antibodies in the ratio to IgM antibodies roughly near to that found in

antibody responses by normal chickens immunized. Also, the early embryonic bursectomy of chickens permitted the differentiation of stem cells into lymphocytes bearing bursa-specific antigen[4]. Accordingly, immunocompetent cells observed in these studies are clearly different from those developed in the chickens treated with TP or the chickens treated with TP and surgically bursectomized observed in the present study.

Chickens treated with TP or those treated with TP and further bursectomized were deprived totally of their immunocompetences by surgical thymus-removal. We should like to add some explanation with regard to the interpretation of this experimental datum. The SRBC, of course, is a thymus-dependent antigen also in the chicken. This has been evidently verified by the previous experiments[5] showing the lack of adoptive immune responses by donor cells treated with anti-thymus cell antiserum in the presence of complement. However, surgical thymectomy of normal chickens in the newly hatched period does not suppress profoundly immune responses against SRBC. The present experiment (Group Con-ST7 in Table 3) showed that surgical thymectomy of normal chickens at 7 days of age caused only a slight reduction in a level of anti-SRBC antibody responses. We have observed such a finding in a number of similar experiments. The finding suggests strongly in the light of well known migration of thymocytes to peripheral lymphoid organs[6] that a considerable number of helper T cells necessary for immune responses against SRBC had already peripheralized before hatching. In contrast, neonatal thymectomy of TP-treated chickens extirpated their capacity to produce anti-SRBC antibodies. Experiments to examine immune responses by chickens treated with TP and neonatally thymectomized were repeated many times. The chickens did not detectable titres of antibodies in every experiment. The two following possibilities are suggestive from this fact: (a) There might be no functional B cells precommited to respond against SRBC with antibody production in the chickens treated with TP and further neonatally thymectomized; (b) The TP-treatment of chickens in embryonic life might delay until 7 days or more after hatching the peripheralization of helper T cells necessary for immune responses against SRBC, which commences before hatching in normal chick embryos. To determine which of the two possibilities is valid, the supplement of the TP12–ST7 or 0TP12–ST7 chickens with thymocytes and thymus-derived cells taken from normal chickens was attempted. The cell transfer, however, did not restore the immunoincompetence of the chickens. Spleen cells taken from the TP12–ST7 chickens together with ABuS-treated spleen cells from normal chickens (as T cell source) were transferred into recipient chickens from which the bursa and the thymus had been removed. However, the cell transplantation did not elicit immune responses in the recipient chickens at all (Table 5). These findings are consistent with the interpretation that the immunoincompetence observed in the TP12–ST7 chickens is attributable to the absence of functional B cells in the chicken. The interpretation suggests strongly that the lack of the development of immunocompetent B cells in the TP-treated chicken is derived from the removal of the thymus, in other words, the development of residual B cells in the TP-treated chicken is dependent upon the thymus. The authors have found no published report with regard to the relation of the B cell development to the thymus except for a paper[7] describing that surgical thymectomy resulted in the decrease in number of lymphocytes in bursal follicles.

Thus, the authors presented here an unusual type of the B cell development in the TP-treated chickens which is relatively independently of the bursa but rather dependent upon the thymus. At the present time our interest is especially focussed on the role of the thymus for generation and maturation of the B cell.

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