[Chem. Pharm. Bull. 30(2) 730—733 (1982)]

Influence of the Administration of Bursa of Fabricius Extract and Anti-bursal Extract Serum on Antibody Formation

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(Received July 7, 1981)

Administration of bursal extract to chickens made immunologically deficient by treatment with cyclophosphamide did not restore immunological competence. Administration of anti-bursal extract serum did not influence antibody formation (agglutinin titre) to sheep red blood cells. These results do not support the presence of hormones, such as bursopoietin, in the bursa of Fabricius.

Keywords——bursa of Fabricius; bursal extract; antibody to bursal extract; cyclophosphamide; anti-bursa serum; Fabricius

Introduction

In chickens, immunoglobulin synthesis and antibody formation are dependent upon the bursa of Fabricius. Suppression of normal bursal development has been shown to cause severe defects in the ontogeny of the humoral immune response.[1] Bursectomy achieved *in ovo* by hormonal means has produced the most severe deficiency in immunologic capabilities.[2] During a search for other agents which could destroy immunocytes, it was found that cyclophosphamide, a potent alkylating agent with known lymphocytotoxicity, is an immunosuppressant in chickens.[3,4] Cyclophosphamide-treated chickens were reported to be functionally and morphologically reconstituted and restored by the injection of bursa cells.[5,6] Bito *et al.* have recently studied the role of the bursa of Fabricius and B-cell development dependent upon the thymus.[7–10]

Meanwhile, it has been reported that the bursal extract contains a lymphocyte-differentiating hormone, bursopoietin,[11] though the physico-chemical properties of bursopoietin have not been reported. We have previously reported some biological properties of an immune-promoting factor in bovine thymus[12] during work on the role of thymus factor in immune regulation. In this paper, we report some basic studies related to the bursa of Fabricius in chickens.

Experimental

Animal——White leghorn chickens were used (Bubcock, Hattori Farm, Nagoya); 5—10 hathced male chickens were used as one group.

Administration of Drugs to Chickens——Testosterone (50 mg), a product of Katayama Chemicals, was dissolved in 0.5 ml of ethanol and 2 ml of 1% sodium alginate was added. Testosterone (2 mg/0.1 ml) was intraperitoneally injected on the day of hatching. Body weight on the day of hatching was 34.8 ± 0.89 g (mean $\pm$ S.E.). Cyclophosphamide, a product of Sigma Chemicals, was dissolved in distilled water at a concentration of 25 mg/ml. This solution (2.5 mg/0.1 ml/chicken) was intraperitoneally injected on the day of hatching and the following day.[6] Control groups were injected with solutions not containing drugs. Water containing polymixin B sulfate (Sigma Chemicals) at a concentration of 10 mg/l was provided as a drinking solution.

Bursal Extract——The bursa (20 g) of Fabricius of 4-week-old male chickens was homogenized in 5 volumes of saline at pH 8.0 and then the solution was centrifuged at $10000 \times g$ for 10 min. The supernatant (0.5 ml/chicken, protein concentration 7.3 mg/ml) was intraperitoneally injected into one-week-old chickens treated or untreated with drugs. The remainder of the supernatant was lyophylized and dissolved at a protein concentration of 70 mg/ml. This concentrated bursal extract (0.5 ml) was also used for injection.

Rabbit Antiserum to the Bursal Extract——The bursa of Fabricius was obtained from 4-week-old chickens. The bursa was homogenized with 5 volumes of saline at pH 8.0. The homogenate was centrifuged at $4000 \times g$ for 10 min and the supernatant was further centrifuged at $100000 \times g$ for 60 min. The supernatant at $100000 \times g$ was used as bursal extract antigen. The extract was emulsified with Freund's complete adjuvant (a product of Iatron, Tokyo) as a w/o type emulsion and then 2 ml of the emulsion was subcutaneously injected to the four pads and the back of each rabbit. Secondary injection was done at 2 weeks after the primary injection. Ten days after the secondary injection, antibody production to the bursal extract was confirmed by the Ouchterlony method. Then the rabbits were bled and anti-bursal extract serum was obtained.

Globulin containing anti-bursal extract antibody was purified from the above anti-bursal serum by fractional precipitation with ammonium sulfate. The serum was diluted 2-fold with physiological saline and the pH was adjusted with ammonium water to 6.8. Saturated ammonium sulfate was added dropwise to the solution with stirring to give 50% saturation. After being mixed for 30 min, the solution was allowed to stand for 30 min and then centrifuged at $13000 \times g$ for 10 min at 15°C. The precipitate was dissolved in a volume of saline equal to that of the initial serum and further precipitated with ammonium sulfate at 50% saturation. After collection of globulin by centrifugation, the precipitate was dissolved in one-fifth volume of saline with respect to the initial serum and the solution was dialyzed against physiological saline. We estimated that the antibody was finally concentrated about 4-fold, and this globulin fraction (31.2 mg/ml) was used as anti-bursal extract antibody.

Control rabbit serum was prepared from the blood of nonimmunized rabbits and globulin fraction was prepared from the serum in the same manner as described above.

Administration of Anti-bursal Extract Serum and Antibody——Anti-serum (0.2 ml/chicken) was intravenously injected on the day of hatching through the wing vein. Anti-bursal extract antibody (0.1 ml/chicken; the quantity of antibody is equivalent to 0.4 ml of antiserum) was injected 3 times, on the day of hatching, and the third day and the fifth day after hatching. In another experiment, daily intravenous injection was done 6 times from the day of hatching to the sixth day. Control globulin or saline was injected on the same schedule in the control group.

Immunization——One ml of 10% sheep red blood cells (SRBC) (2×10^9 cells/ml) was intraperitoneally injected per 100 g body weight of chickens at the ages indicated.

Antibody Titration——Sera were collected from the immunized chickens five days after immunization. Agglutinin to SRBC in sera were measured by microtitration with 20 μl volumes in two-fold dilution with 0.5% SRBC;[7] phosphate-buffered saline was used as a diluent. The plates were incubated at 37°C for 1 h. The titres were expressed as $\log_2$ of the reciprocal of the highest dilution giving positive agglutination reaction and data are shown as mean ± S.E. $\log_2$ titre of the responders. p-Values for differences from the control group were calculated by means of Mann–Whitney's U-test.

Weight of the Bursa of Fabricius and Spleen——After bleeding of the immunized chickens, the bursa and spleen of chickens were dissected out and weighed. The significance of the difference between the data for test and control groups was statistically estimated with Student's t-test.

Plaque-forming Cell (PFC) Assay——PFC-assay of chickens was done with spleen cells of chickens immunized with SRBC according to the published method,[13] modified from the Jerne and Nordin method.[14,15] Briefly, the teased, washed splenocytes obtained on day 5 after immunization with SRBC were mixed with SRBC and layered in 0.7% agar on a base of 1.4% agar. After incubation at 37°C for 30 min, the layer was flooded with 10% solution of guinea pig complements containing rabbit anti-chicken γ-globulin and incubated for an additional 30 min. The hemolytic plaques on the layer were counted and the PFC number was estimated from the cell number of splenocytes.

Other Methods——Electrophoresis of chicken serum on cellulose acetate was carried out according to the previous paper.[16] The stained band (Ponceau 3R) of γ-globulin on cellulose acetate was extracted with 1 ml of 0.01 N NaOH and the optical density at 510 nm was measured. The ratio of γ-globulin to total protein on cellulose acetate was calculated. Protein concentration was determined according to Lowry *et al.*[17]

Results and Discussion

In view of the report that cyclophosphamide-treated chickens were functionally restored by the injection of bursa cells,[5,6] we studied the effect of administration of bursal extract as shown in Table I. Table I shows that administration of cyclophosphamide on the day of hatching decreased the weight of the bursa of Fabricius and the agglutinin titre to SRBC, but did not decrease the body weight or the weight of the spleen. These results suggest that the development of the bursa of Fabricius, but not that of the spleen, is related to the production of agglutinin to SRBC. The second result shown in Table I is that the extracts were not effective for the morphological and functional restoration of the drug-treated chickens

Table I. The Effects of the Bursal Extract on Immunodeficient Chickens

Experiment	Treatment	(n)	Weight (g)			PFC/10^6 cells	Titre of agglutinin	% ratio of globulin[a]
			Body	Bursa	Spleen			
1	Control	6	226±11	1.19±0.16	0.37±0.05	146±73	7.67±0.80	25.9±2.4
	Drugs[b]+saline	7	211±8	0.20±0.06	0.25±0.03	8.1±4.1	3.29±0.66	24.1±2.3
	Drugs[b]+extract[c]	10	192±9	0.17±0.04	0.26±0.02	10.2±5.3	1.20±0.51	21.9±0.9
2	Control	6	307±11	1.93±0.16	0.40±0.06	2.74±0.38	3.17±0.60	13.3±0.8
	Drugs[b]+saline	5	251±20	0.34±0.10	0.26±0.04	0.92±0.28	1.40±0.51	15.9±1.6
	Drugs[b]+conc. extract[d]	5	267±13	0.32±0.07	0.40±0.05	0.87±0.16	0.60±0.24	16.0±1.8

a) Ratio to total protein.
b) Cyclophosphamide 2.5 mg/chicken and testosterone 2 mg/chicken.
c) The amount of protein extract was 3.6 mg/0.5 ml/chicken.
d) The amount was 36.5 mg/0.5 ml/chicken.

in terms of the weight of the bursa, the PFC number, or the agglutinin titre. These results suggests that the differentiation of bone marrow stem cells to mature B cells does not depend on a soluble factor in the bursa organ but on cell-mediated type regulation. However, further work is necessary to confirm this, because it is possible that the factors are labile and are inactivated during preparation of the bursal extract, or that the amount of the bursal extract used does not contain the minimum effective dose. Brand *et al.* showed the presence of bursopoietin in *in vitro* experiments[11] not by *in vivo* administration.

Table II. Results of Administration of Bursal Extract Antibody to Newly Hatched Chickens

Experiment	Treatment	Weight			Titre of agglutinin	(n)
		Body (g)	Bursa (g)	Spleen (mg)		
1[a]	None	125±3.3	0.38±0.17	107±6.4	3.67±2.18	9
	Saline	129±3.9	0.36±0.03	106±7.3	2.57±1.30	8
	Control serum	113±2.4	0.34±0.02	106±10.2	3.04±0.44	7
	Antiserum	110±4.9	0.32±0.04	92±9.9	2.92±0.88	9
2[b]	Control globulin	142±2.4	0.58±0.02	155±11.8	2.67±0.49	6
	Antibody	140±6.9	0.54±0.06	161±15.7	2.43±0.61	7
3[c]	Saline	166±3.8	0.80±0.04	217±13	7.20±1.05	10
	Control globulin	164±3.0	0.83±0.04	211±13	5.90±1.00	10
	Antibody	167±2.6	0.76±0.03	221±31	5.80±1.20	10

a) Antiserum was injected once on the day of hatching. Antigen was injected on day 14.
b) Antibody was injected three times, on the first day of hatching, and the third day and the fifth day after hatching. Antigen was injected on day 18.
c) Antibody was injected daily six times from the day of hatching through the sixth day. Antigen was injected on day 17.

Next, we studied the effect of administration of anti-bursal extract antibody. If the bursa of Fabricius secreted some immune-promoting factor for the development of stem cells to mature B cells, antiserum to the bursal extract might influence the agglutinin titre to SRBC. Table II shows the results of injection of antibody to the bursal extract (the supernatant at 100000×***g***). No effect of administration of antibody to the bursal ecxtrat could be observed as shown in Table II. In particular, in experiment 3 in Table II, antibody to the bursal extract was injected daily from the day of hatching to the sixth day after hatching but even in this case no significant difference between the test and control groups could be seen

in terms of the weight of the bursa of Fabricius or the agglutinin titre. Antibody to the bursal extract was injected intravenously, so the absence of any effect of this treatment indicates that chicken serum does not contain any factor which is influenced by anti-bursal antibody.

The results of administration of the bursal extract and anti-bursal extract serum suggest that development of stem cells derived from bone marrow to mature B cells is dependent upon a cell-mediated process, not a hormonal process. However, our experiments are preliminary and the conclusion that bursal hormones are not present requires confirmation. In this work, we found that the weight of the bursa of Fabricius was decreased by injection of cyclophosphamide. Similar results were reported by Romppanen *et al.*[18] during the preparation of this manuscript.

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