Evaluation of Effects of Bursa Fabricius Extracts on Development of Immunocompetence in Neonatally Bursectomized Cockerels[1] (34923)

NANCY E. GOEKEN, ROBERT P. BREITENBACH, AND JAMES T. BARRETT

Zoology Department and Microbiology Department, University of Missouri, Columbia, Missouri 65201

Since the discovery of the immunological function of the bursa of Fabricius in the bird in 1956 (1), many investigators have sought to elucidate the mechanism of its action. It has been well established that the bursa functions as a primary rather than a secondary lymphoid organ and that it influences circulating antibodies but not cellular immunity (2). However, debate has continued as to whether the bursa secretes some circulating humoral factor which promotes the differentiation and proliferation of antibody producing cells in the peripheral lymphoid tissues; or whether cells are produced or transformed *in situ* in the bursa, and then seeded to the periphery; or whether some combination of these two mechanisms occurs.

The hormonal hypothesis has been tested by several investigators by using bursal grafts (3) or bursal implants in "cell-free" Millipore chambers (4–6). These have enhanced the immune response of bursectomized chickens, thus providing some evidence for a humoral factor from the bursa. However, the reader must be reminded that the bursa is a diverticulum of the gut and has a rich flora. Dent *et al.* (7) have recently shown that gut, which has a normal flora similar to bursa, has just as great an effect when implanted in one of these chambers. This suggests that the enhanced immune responses following bursal grafts or implants may be an adjuvant effect of foreign tissue, bacteria, or bacterial toxins. It has also been demonstrated by Capalbo *et al.* (8) that filter porosities of 0.45 μ or greater do not exclude cells of the peritoneal cavity. Thus, cell to cell interactions cannot be ruled out in previous Millipore chamber experiments.

Reports on the effects of cell-free bursal extracts have been somewhat contradictory. Glick (9) and Jankovic *et al.* (10) found that they increased the immune response. However, control extracts of muscle and liver, respectively, also produced a similar increase. This again indicates a nonspecific adjuvant effect. Takahashi (11) reported an enhancement of the immune response following three intraperitoneal injections of bursal extract at 48-hr intervals in surgically bursectomized but not in hormonally bursectomized chicks. Extracts of thymus had no effect. No attempts were made, however, to insure a bacterium-free extract, so the adjuvant effect cannot be excluded. Edwards *et al.* (12) have reported no increase, but their result can be validly questioned on the basis of developmental "competence." Their donors and recipients were not the same age and also their birds received the extract for a relatively short period of time. Thus, the quality of their extract, duration of treatment, and the competence of the recipient cells to respond are all subject to doubt.

The following experiment was designed to eliminate the problems of sterility, differences in age of donor and recipient, and length of treatment; all of which make clear interpretation of previous work difficult.

Materials and Methods. Day old White Leghorn cockerels were divided into 4 groups. Three groups were surgically bursectomized by day 3 post-hatch. The fourth group was sham bursectomized with a full surgical opening on day 4.

The shams (S) were untreated following surgery and the bursectomized birds were randomly divided into three groups. One bursectomized groups was not treated further following surgery (Bx), another received bur-

[1] Supported in part by USPH Grant AI-05643.

sal extract (BE), and the third received pancreas extract (PE) as a tissue control.

Extracts of bursa and pancreas were prepared in the following manner. One g of tissue was minced in 3 ml of ice cold 0.01 *M* phosphate buffered saline at pH 7.3 and then homogenized. Hand homogenization was used to prevent the destruction of bacteria and the release of their toxins. This material was then centrifuged twice at 25,000 rpm for 20 min in a model L Spinco ultracentrifuge. The resulting supernatants of bursa and pancreas had approximately the same protein concentration (20 mg/ml). These supernatants were immediately filtered thru a 0.45 μ bacterial filter and stored frozen in sterile containers until used.

To insure that donors and recipients would be at the same stage of development, organ extracts were prepared fresh every 3 to 4 days during the period of treatment. In each case donor birds were of the same age as recipients. This experimental design takes competence into account and more closely approximates the normal developmental situation than the work previously cited.

Treated birds received 0.25 ml of extract daily, subcutaneously, from days 7 thru 14, and 0.1 ml of extract from days 15 thru 30. In order to determine the immediate effects of the procedure, five birds from each group were necropsied on day 30. Weights of spleen, pancreas, and thymus were taken and expressed as percentage of body weight; and spleen, thymus, and cecal tonsils were prepared for histological examination by fixation in Bouin's solution and staining with Schorr's modified procedure. The number of splenic bursal dependent centers per 40× field was determined by averaging the values from three different sections from each spleen.

To improve the comparative value of the control groups, the previously sham bursectomized (S) birds were bursectomized on the same day (day 31) that tissue extract injections were stopped in the other groups.

Both primary and secondary responses to bovine serum albumin (BSA) and *Brucella abortus* were determined. All birds received 1 ml of commercial formalinized *B. abortus* antigen intravenously on days 41 and 51. Blood was drawn by cardiac puncture on days 48 and 58. The sera were harvested and frozen until antibody determinations were made. Antibody titers were determined by an agglutination test using a 2-fold antiserum dilution series. Commercial *B. abortus* antigen and varying concentrations of antiserum were combined in equal amounts in 0.9% NaCl. The end point was taken as the highest dilution of antiserum which produced a positive agglutination after incubation at 37° for 24 hr and 4° overnight.

All groups received 100 mg of BSA intravenously on days 59 and 70. Blood was drawn on days 66 and 77. Titers were determined by a precipitin test using an antiserum dilution series. BSA (5 mg/100 ml) and varying concentrations of antiserum were combined in equal amounts in 9.0% NaCl. The end point was taken as the highest dilution of antiserum which produced a positive precipitin reaction after incubation overnight at room temperature.

The remaining chickens were killed on day 77 by using an overdose of Nembutal and tissues were treated as on day 30.

Results. The primary and secondary responses to *B. abortus* antigen showed no differences among the bursectomized groups, but the responses of all three of these groups were significantly lower than those of sham operated birds (see Table I). The same rela-

TABLE I. Antibody Response to *B. abortus*.[a]

Group:	BE	PE	Bx	Sham
A. Primary response				
$\bar{x}$	4.1^{A}	3.4^{B}	3.6^{C}	7.7^{ABC}
Total *n*	7	7	8	7
No. responding	7	7	8	7
% of Sham	53.2	44.2	46.8	—
B. Secondary response				
$\bar{x}$	3.6^{A}	4.0^{B}	5.0^{C}	7.6^{ABC}
Total *n*	8	5	5	5
No. responding	8	5	5	5
% of Sham	47.3	52.6	65.8	—

[a] The reciprocal of each antibody titer was converted to $\log_2 \times 10 + 1$. Groups which have the same superscript differ significantly at the 95% significance level, according to Duncan's Multiple Range Test.

TABLE II. Antibody Response to BSA.[a]

Group:	BE	PE	Bx	Sham
A. Primary response				
$\bar{x}$	0^A	0^B	0^C	4.7^{ABC}
Total	7	6	7	6
No. responding	0	0	0	6
% of Sham	0	0	0	—
B. Secondary response				
$\bar{x}$	0.86^A	0.71^B	1.83^C	5.5^{ABC}
Total n	7	7	6	6
No. responding	2	2	3	6
% of Sham	15.6	12.9	33.3	

[a] The reciprocal of each antibody titer was converted to $\log_2$; the means and statistical analysis were calculated using 0 as the value for the negative sera. Groups which have the same superscript differ significantly at the 95% significance level according to Duncan's Multiple Range Test.

tionship was demonstrated in the response to BSA (see Table II).

The only significant autopsy finding was that by day 77 all three initially bursectomized groups had body weights which were significantly lower than those of the sham operated group (BE, 782 g; PE, 832 g; Bx, 811 g; and S, 968 g. $p < 0.05$).

No significant differences in the numbers of splenic bursal dependent centers were found among the groups on either day 30 or 77. Also, no differences were found in the microscopic appearance of the thymus or cecal tonsils on day 30 or 77.

Discussion. We believe that our experiment has accomplished the following: eliminated the presence of bacteria or bacterial toxins as adjuvants, provided bursal extract to recipients over a significant time interval during their development, and provided extract from donors of the same hatch (age and strain). This procedure should have assured that any product produced by the bursa at any point in time was delivered to potential target cells during the period of their competence to respond to that product. Also, the experimental design (maintenance of tissues and extracts at low temperatures, and frequent preparations of fresh extracts) minimizes the possibility that the bursal factor was denatured. It is possible that the amount of the factor produced *in vivo* exceeds the amount administered. However, repeated daily injections greatly reduce the probability that an insufficient amount was administered.

Treatment with bursal extract did not repair the effects of bursectomy upon the immune response. The response to BSA, a soluble antigen, was much more severely depressed than the response to *B. abortus*, a particulate antigen; confirming the previous findings (13, 14) that bursectomy influences the production of IgG to a greater extent than IgM.

All bursectomized groups showed a weak, but nevertheless detectable, secondary response to BSA. This result is in agreement with prior evidence indicating that bursectomized birds can demonstrate immunological memory (15). Apparently, if any primary response to the antigen can be elicited, the anamnestic response is normal. This would be compatible with a model in which the number of antigen reactive or antibody producing cells is severely reduced, but in which those present do function normally. There is no secondary response to *B. abortus*, per se, in any of the experimental groups. This is in accordance with the fact that an anamnestic response may not be seen in IgM production (16). It reaches approximately the same level with each exposure to antigen.

The lack of differences in the appearance of thymus and cecal tonsils between sham and bursectomized birds is similar to findings previously reported for surgically bursectomized birds (3) except that those investigators did find a plasma cell depletion in the cecal tonsils. Apparently, the removal of the bursa after hatching may not consistently produce cellular defects of a magnitude that can be distinguished microscopically.

On the basis of these data and previous findings, we must conclude that little experimental evidence exists to support the hypothesis that the bursa of Fabricius produces a humoral factor. However, evidence does exist to support the concept that the bursa is a type of cell processing center similar to the mammalian thymus. Woods and Linna (17) have demonstrated the transport of cells from the bursa to peripheral tissues, especially spleen and thymus, and other workers have

shown that there is a high rate of migration of cells into the bursa (18, 19).

There has been a trend to accept the meager and questionable results of extract and implant experiments as conclusive evidence for the existence of a humoral function of the bursa of Fabricius in immune processes. We believe that our data permit us to challenge such acceptance. We would conclude that while a humoral contribution by the bursa has not been disproven, a model wherein cells are continually being processed and seeded to the periphery better explains the existing experimental evidence.

Summary. This experiment was designed to serve as a careful test of the hypothesis that the bursa of Fabricius produces a hormone responsible for the development of immunocompetence. Cell- and bacterium-free extracts of the bursa or pancreas were administered subcutaneously for 23 days to neonatally bursectomized cockerels. Immune responses were tested at 30 and 77 days of age. The bursal extracts produced no significant repair of the immuno-deficient state. The responses to BSA and *B. abortus* antigens did not differ in bursectomized birds receiving bursal or pancreas extract. However, all bursectomized birds showed significantly lower primary and secondary responses than did sham operated birds. No significant differences were measured in the number of splenic bursal dependent centers between bursectomized birds receiving bursal extract and their controls. On the basis of our findings, we conclude that prior evidence for a humoral function of the bursa should be challenged.

1. Glick, B., Chang, T. S., and Jaap, R. G., Poultry Sci. **35**, 224 (1956).

2. Cooper, M. D., Peterson, R. D. A., and Good, R. A., Nature (London) **205**, 143 (1965).

3. Isakovic, K., and Jankovic, B. D., Int. Arch. Allergy **24**, 296 (1964).

4. Jankovic, B. D., and Leskowitz, S., Proc. Soc. Exp. Biol. Med. **118**, 1164 (1965).

5. St. Pierre, R. L., and Ackerman, G. A., Science **147**, 1307 (1965).

6. May, J. D., Hill, C. H., and Garren, H. W., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **26**, 769 (1967).

7. Dent, P. B., Perey, D. Y. E., Cooper, M. D., and Good, R. A., J. Immunol. **101**, 799 (1968).

8. Capalbo, E. E., Albright, J. F., and Bennett, W. E., J. Immunol. **92**, 243 (1964).

9. Glick, B., Poultry Sci. **39**, 1097 (1960).

10. Jankovic, B. D., Isakovic, K., and Horvat, J., Experentia **23**, 1062 (1967).

11. Takahashi, K., Jap. J. Microbiol. **11**, 1 (1967).

12. Edwards, J. L., Zawrocka-Wrzolek, T., and Kreps, J. S., Lab. Invest. **19**, 627 (1968).

13. Arnason, B. G., and Jankovic, B., J. Immunol. **99**, 917 (1967).

14. Ortega, L. G., and Der, B. K. Fed. Proc., Fed. Amer. Soc. Exp. Biol. **23**, 546 (1964).

15. Jankovic, B. D., and Isakovic, K., Nature (London) **211**, 202 (1966).

16. Boyd, W. C., "Fundamentals of Immunology," 773 pp. Wiley (Intersciences, New York (1966).

17. Woods, R., and Linna, J., Acta Pathol. Microbiol. Scand. **64**, 470 (1965).

18. Moore, M. A. S., and Owen, J. J. T., Develop. Biol. **14**, 40 (1966).

19. Jaffee, W. P., and Fechheimer, N. S., Nature (London) **212**, 92 (1966).

Received Feb. 16, 1970. P.S.E.B.M., 1970, Vol. 134.