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STUDIES ON THE BURSA OF FABRICIUS AND ITS ROLE IN THE IMMUNE RESPONSE IN CHICKENS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By


* * * * * *

The Ohio State University
1965

Approved by

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CONTENTS

ACKNOWLEDGMENTS ........................................ 11
VITA ....................................................... iii
TABLES ..................................................... vi
ILLUSTRATIONS .......................................... vii
GENERAL INTRODUCTION .................................... 1

PART I. IMMUNOLOGICAL RESPONSE OF BURSECTOMIZED
CHICKENS FOLLOWING IMPLANTATION OF
THE BURSA OF FABRICIUS

Materials and Methods ..................................... 5
Results .................................................... 10
Discussion ................................................... 17
Summary .................................................... 20

PART II. RE-ESTABLISHMENT OF "BURSA-DEPENDENT
FOLLICLES" IN THE SPLEENS OF BURSECTOMIZED
CHICKENS BY IMPLANTATION OF DONOR BURSA

Materials and Methods ..................................... 22
Results .................................................... 26
Discussion ................................................... 37
Summary .................................................... 42

PART III. TRANSFER OF IMMUNOLOGICAL REACTIVITY

Materials and Methods ..................................... 45
Results .................................................... 48
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discussion</td>
<td>53</td>
</tr>
<tr>
<td>Summary</td>
<td>58</td>
</tr>
<tr>
<td>GENERAL SUMMARY</td>
<td>60</td>
</tr>
<tr>
<td>APPENDIXES</td>
<td>63</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>75</td>
</tr>
</tbody>
</table>
Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Antibody Production in Bursectomized and Control Chickens, and the Effect of Bursa Implants on Antibody-Producing Deficiency in Bursectomized Chickens</td>
<td>10</td>
</tr>
<tr>
<td>2. Electrophoretic Distribution of Serum Protein Components from Bursectomized Chickens Implanted with Bursa-Filled Diffusion Chambers</td>
<td>14</td>
</tr>
<tr>
<td>3. Cytological Analysis of Selected Red Pulp Areas of the Spleens of Control, Bursectomized and Bursectomized-Bursa Implanted Chickens</td>
<td>29</td>
</tr>
<tr>
<td>4. Mean Number of Lymphocytic Nodules of the Spleens of Control, Bursectomized and Bursectomized-Bursa Implanted Chickens</td>
<td>30</td>
</tr>
<tr>
<td>5. Scheme of Lymphocytic Organ Transfer to Bursectomized Chickens</td>
<td>51</td>
</tr>
<tr>
<td>6. Antibody Production in Bursectomized and Control Chickens, and the Effect of Lymphocytic Organ Transplant on Antibody-Producing Deficiency in Bursectomized Chickens</td>
<td>52</td>
</tr>
</tbody>
</table>
### ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Scheme of implantation of seven-day-old, bursectomized chicks containing bursa-filled diffusion chamber, bursa alone, and empty diffusion chamber</td>
<td>8</td>
</tr>
<tr>
<td>2. Mean titers of antibody in (1) non-bursectomized control chickens; (2) hormonally bursectomized chickens containing bursa; (3) hormonally bursectomized chickens containing bursa-filled diffusion chambers; and (4) hormonally bursectomized chickens containing empty diffusion chambers</td>
<td>12</td>
</tr>
<tr>
<td>3. Percentages of gamma-globulin present in the immune serum of (1) non-bursectomized control chickens; (2) hormonally bursectomized chickens containing bursa; (3) hormonally bursectomized chickens containing bursa-filled diffusion chambers; and (4) hormonally bursectomized chickens containing empty diffusion chambers</td>
<td>16</td>
</tr>
<tr>
<td>4. Two lymphocytic nodules (ln) adjacent to a single artery (a) with a Schweigger-Seidel sheath (ss) in the red pulp from the spleen of a control chicken</td>
<td>28</td>
</tr>
<tr>
<td>5. Two lymphocytic nodules (ln) from the spleen of a control chicken</td>
<td>28</td>
</tr>
<tr>
<td>6. Single lymphocytic nodule (ln) from the spleen of a control chicken adjacent to an artery</td>
<td>28</td>
</tr>
<tr>
<td>7. Single lymphocytic nodule (ln) from the spleen of a control chicken showing capsular membrane (m) and two plasma cells (p) in the adjacent red pulp</td>
<td>28</td>
</tr>
<tr>
<td>8. Spleen from bursectomized chicken showing diffuse lymphocytic area corresponding to the white pulp</td>
<td>32</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>9. Spleen from bursectomized chicken showing diffuse lymphocytic area (dl) and Schweigger-Seidel sheaths (ss)</td>
<td>32</td>
</tr>
<tr>
<td>10. Spleen from bursectomized chicken with diffuse lymphocytic area (dl) and adjacent artery (a)</td>
<td>32</td>
</tr>
<tr>
<td>11. Spleen from bursectomized chicken showing lymphocytic area (dl) surrounding an artery</td>
<td>32</td>
</tr>
<tr>
<td>12. Single lymphocytic nodule (ln) associated with artery (a) from spleen of bursectomized chicken implanted with bursa alone</td>
<td>36</td>
</tr>
<tr>
<td>13. Single lymphocytic nodule (ln) from spleen of bursectomized chicken implanted with a bursa-filled diffusion chamber</td>
<td>36</td>
</tr>
<tr>
<td>14. Single lymphocytic nodule (ln) from the spleen of a bursectomized chicken implanted with bursa-filled diffusion chamber</td>
<td>36</td>
</tr>
<tr>
<td>15. Single lymphocytic nodule from spleen of bursectomized chicken implanted with bursa-filled diffusion chamber showing &quot;blast&quot; cell in the vascular channel adjacent to capsule of the nodule</td>
<td>36</td>
</tr>
<tr>
<td>16. Mean titers of antibody production in (1) hormonally bursectomized chickens implanted with bursa followed by injection of antigen; (2) non-bursectomized control chicks injected with antigen; (3) hormonally bursectomized chicks implanted with spleen followed by injection of antigen; and (4) hormonally bursectomized chicks not implanted, but injected with antigen</td>
<td>50</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION

Until recently the structure and function of the bursa of Fabricius has remained relatively obscure since the original description by Hieronymus Fabricius (published posthumously in 1621). Fabricius stated that this organ, found only in the female, was a double sac which opened between the cloaca and the uterus to serve as a reservoir for sperm introduced by the male (4). Later investigators found that this organ, present only in birds, was present in both sexes, was not a double sac and had no connection with the uterus. Extensive historical reviews of the bursa of Fabricius have been presented by Arvy (5), Glick (21), and Warner and Szenberg (60).

The demonstration that surgically bursectomized chickens had greatly diminished capacity to produce antibody (22) and the observation that 19-nortestosterone inhibited the normal development of the bursa with associated immunological deficiency (40, 48), stimulated research which resulted in a growing body of evidence to indicate the important role of the bursa of Fabricius in the development of immunological responsiveness. Renewed interest in research regarding the bursa of Fabricius followed the investigation showing the epithelial origin of lymphocytes in the bursa (1) and the
similar observation that lymphocytes were derived from the epithelial primordia of the developing thymus (8).

At the present time the role of the bursa of Fabricius is considered to be confined to development of the potential to form antibody, the mechanism of which is poorly understood (7, 12, 21, 22, 24, 26, 38, 39, 48, 50). In contrast, the thymus is considered responsible for the direct cellular response to tissue graft, i.e., homograft rejection, in the chicken (6, 7, 24, 26, 41, 53, 58, 59, 61). Thus, the thymus and the bursa of Fabricius work together to establish total immunological responsiveness in the chicken (17, 53, 59, 61).

There are four possible mechanisms by which the bursa confers the capacity to form antibodies: (1) the bursa may serve as a site for the production of antibody (31); (2) the bursa may play a purely cellular role by producing immunologically competent lymphocytes which migrate from the bursa to populate other lymphocytic organs (55); (3) the bursa may exhibit a hormonal or hormone-like function in the formation of antibody (1, 10, 22, 27, 28); and (4) the bursa may exhibit both a cellular and a hormonal function (27, 28).

The spleen appears to be the primary site for the elaboration of antibody (35, 36, 49), while the role of the bursa in the production of antibody is indirect, but absolutely essential (17, 20, 49). Since discrete lymphocytic follicles and plasma cells present in the spleens of normal
chickens are not present in the spleens of bursectomized birds, these elements appear to constitute a bursa-dependent system and have a role in the immune mechanism (17).

This investigation has been undertaken in order to clarify further the role of the bursa of Fabricius in the immunological response of the chicken. Specifically, this study will attempt to (1) determine the presence or absence of a non-cellular humoral substance derived from the bursa of Fabricius capable of restoring immunological reactivity in bursectomized chickens; (2) determine whether bursa-dependent lymphocytic follicles and plasma cells can be re-established in the spleens of bursectomized chickens by implantation of bursa from normal donors; and (3) evaluate the potential of lymphocytic organs, viz., thymus, spleen, and bursa of Fabricius, to transfer the ability to produce antibody from immunologically reactive chicken donors to recipients rendered immunologically inert by testosterone bursectomy.
PART I

IMMUNOLOGICAL RESPONSE OF BURSECTOMIZED CHICKENS FOLLOWING IMPLANTATION OF THE BURSA OF FABRICIUS
MATERIALS AND METHODS

The fertile eggs and the chickens used throughout this study were from the regional, random-bred White Leghorn population obtained from the Department of Poultry Science of The Ohio State University. Complete details of each of the procedures have been described in the appendixes and will be briefly described here.

Bursectomy

The fertile eggs were divided into two groups: (A) One group of eggs (experimental) was given a 0.1 ml. (2.5 mg.) injection of testosterone propionate in sesame oil (Schering) on the fifth day of incubation. Administration of testosterone or its derivatives on the fifth day of incubation causes the complete inhibition of the bursa of Fabricius (23, 38, 40, 48, 57) with no possible chance for proliferation of lymphocytes or stromal cells (1, 2, 3). (B) A second group of eggs, not treated with testosterone, served as a source of normal bursas for subsequent implantation as well as controls, i.e., animals with intact bursas and having normal immunological reactivity. All chickens were hatched and maintained under the standard conditions and diet as employed by the Department of Poultry Science.
Implantation of bursa

The bursas used for implantation were obtained from chicks seven days after hatching. Bursas were removed surgically, placed in Hank's Balanced Salt Solution, and divided into four equal pieces. A piece of bursa was surgically implanted either subcutaneously or intraperitoneally into one group of seven-day-old, testosterone-bursectomized chicks. Another group of seven-day-old, testosterone-bursectomized chicks was surgically implanted subcutaneously or intraperitoneally with a bursa enclosed within a Millipore diffusion chamber. Millipore diffusion chambers were constructed of two 25 mm. plastic cellulose filters of 0.45 micra porosity (Millipore Filter Company, Bedford, Massachusetts) and sealed with MF Cement Formulation No. 2. In comparative studies, filters of a smaller porosity, 0.1 micra, were used. As a control, hormonally bursectomized chicks were implanted with empty diffusion chambers (Figure 1).

Immunization and agglutination tests

On the ninth day after hatching, i.e., one day following the surgical procedures, chicks from the control and experimental groups were given a 1.0 ml. intramuscular injection of killed Salmonella typhimurium, standardized at $3 \times 10^9$ cells/ml. Four weeks later, a second injection of S. typhimurium was given. Two weeks after the second injection of antigen, blood samples were obtained by cardiac puncture.
Figure 1
Scheme of implantation of seven-day-old, bursectomized chicks containing bursa-filled diffusion chamber, bursa alone, and empty diffusion chamber.
BURSA
MILLIPORE FILTER
MILLIPORE FILTER
BURSA
MILLIPORE FILTER
7 DAY CHICK

1
The blood was placed in centrifuge tubes and allowed to clot at room temperature. The serum was removed from the clot, centrifuged and stored at 4°C. Agglutination tests were made by adding 0.25 ml. of the standard antigen to 0.25 ml. of the serum in serial dilution from 1:2 to 1:1024. The tubes were incubated at 45°C for two hours, then refrigerated for 24 hours and antibody titers were determined. Agglutination above a titer of 1:4 was considered to be positive, i.e., indicating the presence of antibody to S. typhimurium. Agglutination was graded subjectively from 4+ to 0. The end point of antibody activity was taken as the last tube showing 1+ agglutination. The antibody titers have been expressed as arithmetic means.

**Electrophoresis**

Serum electrophoresis patterns were obtained by placing 0.006 ml. of serum on S and S 2043A paper strips and immersing the strips in a Spinco Duostat containing veronal acetate buffer for 16 hours. The strips then were dried for 30 minutes in an oven at 37°C, pre-rinsed in 1000 ml. of absolute methanol for six minutes and stained with 1 per cent bromphenol blue in absolute methanol. After three rinses for six minutes each in 5 per cent glacial acetic acid, the strips were oven-dried for ten minutes and exposed to ammonia vapor for two hours. Electrophoresis patterns or diagrams of serum protein were obtained by putting the strips through a Beckman Model R Spinco Analytrol.
RESULTS

Antibody production in normal and bursectomized chickens

Non-bursectomized control chickens produced a high titer of antibody to S. typhimurium in 22 of 30 birds with a mean antibody titer of 1:330. By direct contrast, all (10 of 10) testosterone-bursectomized chickens containing empty diffusion chambers were unable to produce demonstrable antibody. The results of antibody production in normal and bursectomized chickens have been summarized in Table 1 and Figure 2.

TABLE 1

ANTIBODY PRODUCTION IN BURSECTOMIZED AND CONTROL CHICKENS, AND THE EFFECT OF BURSA IMPLANTS ON ANTIBODY-PRODUCING DEFICIENCY IN BURSECTOMIZED CHICKENS

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Birds</th>
<th>Range of Titer</th>
<th>Mean Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-bursectomized control</td>
<td>22/30</td>
<td>1:256 - 1:512</td>
<td>1:330</td>
</tr>
<tr>
<td>Bursectomized with empty diffusion</td>
<td>10/10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>chamber implanted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bursectomized with bursa-filled</td>
<td>29/41</td>
<td>1:64 - 1:256</td>
<td>1:105</td>
</tr>
<tr>
<td>diffusion chamber implanted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bursectomized with bursa alone</td>
<td>15/20</td>
<td>1:64 - 1:128</td>
<td>1:115</td>
</tr>
<tr>
<td>implanted</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2

Mean titers of antibody production in (1) non-bursectomized control chickens; (2) hormonally bursectomized chickens containing bursa; (3) hormonally bursectomized chickens containing bursa-filled diffusion chambers; and (4) hormonally bursectomized chickens containing empty diffusion chambers.
The effect of bursa implantation upon antibody production in bursectomized chickens

As compared with bursaless chickens, implantation of bursa pieces resulted in formation of antibody in 15 of 20 bursaless chickens with a mean titer of 1:115. Implantation of bursa enclosed within Millipore diffusion chambers reconstituted production of antibody in 29 of 40 hormonally bursectomized chickens, which showed a mean antibody titer of 1:105. The bursectomized chickens rejected the implanted grafts with great uniformity, and no demonstrable bursa graft was recovered at the end of the seventh week, i.e., termination of the experimental procedure. No differences were noted in the antibody titer of animals receiving grafts implanted subcutaneously or intraperitoneally. Among the hormonally bursectomized chickens grafted with bursa-filled diffusion chambers, no differences were noted in the production of antibody when chambers were constructed from 0.45 micra or 0.1 micra porosity filters. The results of bursa grafting upon antibody production have been summarized in Table 1 and Figure 2.

Electrophoretic findings

The electrophoretic patterns of the protein components of serum have been listed in Table 2 and are graphically represented in Figure 3. The percentages of alpha and gamma-globulins were greater in the unoperated, control
chickens than in the bursectomized animals containing empty diffusion chambers. The sera from bursectomized chickens grafted with a piece of bursa and from bursectomized birds grafted with bursa-filled diffusion chambers had higher alpha and gamma-globulin percentages than the bursectomized chickens containing empty diffusion chambers, but the percentages were still less than the non-bursectomized control birds.

TABLE 2

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<thead>
<tr>
<th>Group</th>
<th>Serum Protein Components (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Albumin</td>
</tr>
<tr>
<td>Non-bursectomized control</td>
<td>33.3</td>
</tr>
<tr>
<td>Bursectomized with empty diffusion chamber implanted</td>
<td>51.0</td>
</tr>
<tr>
<td>Bursectomized with bursa-filled diffusion chamber implanted</td>
<td>42.2</td>
</tr>
<tr>
<td>Bursectomized with bursa alone implanted</td>
<td>34.3</td>
</tr>
</tbody>
</table>
Figure 3

Percentages of gamma-globulin present in the immune serum of (1) non-bursectomized control chickens; (2) hormonally bursectomized chickens containing bursa; (3) hormonally bursectomized chickens containing bursa-filled diffusion chambers; and (4) hormonally bursectomized chickens containing empty diffusion chambers.
GAMA GLOBULIN

TYPE OF IMPLANT

% GAMMA GLOBULIN

TYPE OF IMPLANT

1

2

3

4
DISCUSSION

This study has indicated that the implantation of grafts of the bursa of Fabricius enclosed within cell-impermeable Millipore diffusion chambers reconstituted immunological reactivity in bursectomized chickens. The data presented suggests strongly that the bursa of Fabricius elaborates a non-cellular (hormone-like) substance which enhances immunological reactivity in the chicken. Serum electrophoresis determinations added confirmation to this finding in that the hormonally bursectomized chickens had markedly decreased amounts of alpha and gamma-globulin, in agreement with reports for bursectomized chickens which received no further treatment (10, 33). The bursectomized chickens containing bursa-filled diffusion chambers showed alpha and gamma-globulin concentrations greater than the bursectomized chickens, although the concentrations were less than normal. The alpha and gamma-globulin components of chicken serum contain the antibody of the immune serum (9).

No morphological evidence of the bursa of Fabricius was found upon recovery of the bursa-filled diffusion chamber grafts at the seventh week, i.e., termination of the experimental procedures. The absence of viable tissue would indicate that the bursa was not the site of antibody production.
as has been suggested by previous investigators (31). This suggestion was based upon the observation of antibody-producing cells in the bursa using fluorescent-antibody technique after multiple injection of antigen. The observation that surgical bursectomy ten weeks after hatching failed to influence subsequent antibody formation further argues against the bursa being a major site for the production of antibody (12, 22).

Thorbecke et al. (60) have made the suggestion that the bursa may not be a site of antibody formation, but a place where inductor cells are formed. These inductor cells would be capable of inducing production of antibody from functionally-undifferentiated cells of lymphocytic tissues in other regions of the body. The present study indicated that the antibody-producing deficiency exhibited by hormonally bursectomized chickens can be reconstituted to a significant degree by implants of bursa from donor animals. This observation is in agreement with the recent studies of Isakovic and Jankovic (27, 28). It is possible that there had been a migration of cells from the implanted graft of bursa to some other lymphoid region of the chicken. In order to rule out the migration of cells from the bursa, grafts were enclosed in cell-impermeable Millipore diffusion chambers as had been employed recently in similar studies of the mammalian thymus (32, 45, 64). All migration of cells can be prevented if tissues are placed in diffusion chambers.
constructed of Millipore filters having a porosity of 0.45 micra or smaller (51). The finding that bursectomized chickens implanted with bursa-filled diffusion chambers were able to produce antibodies indicates that the bursa of Fabricius produces a diffusable, non-cellular substance which establishes immunological reactivity in the chicken. This has been suggested by other investigators (10, 27, 28) and is in agreement with the observation of Glick (20), who found that injected saline extracts of acetone-dried bursas enhanced antibody production in surgically bursectomized chickens. The evidence presented in this study utilizing hormonally bursectomized birds is more conclusive, since there could have been no residual bursa in the grafted bird due to the in ovo injection of testosterone which results in total bursectomy.
SUMMARY

The bursa of Fabricius is a lympho-epithelial organ peculiar to birds which is essential for the production of antibody. The production of antibody to *S. typhimurium* has been studied in White Leghorn chickens which were (1) hormonally bursectomized; (2) hormonally bursectomized and grafted with a donor bursa; and (3) hormonally bursectomized and grafted with bursa enclosed within cell-impermeable Millipore diffusion chambers. The results obtained indicate that (1) hormonally bursectomized chickens are unable to form antibodies to *S. typhimurium*; (2) as compared with bursectomized birds, grafts of bursa and bursa enclosed within Millipore diffusion chambers reconstitute the production of antibody in hormonally bursectomized chickens and enhance the concentrations of alpha and gamma-globulin components in the immune serum.

The evidence for the existence of a non-cellular (hormone-like) substance elaborated by the bursa of Fabricius which establishes immunological reactivity in the chicken is strongly suggested by the observation that grafts of bursa enclosed within cell-impermeable Millipore diffusion chambers reconstitute production of antibody in bursectomized chickens.
PART II

RE-ESTABLISHMENT OF "BURSA-DEPENDENT FOLLICLES" IN THE SPLEENS OF BURSECTOMIZED CHICKENS BY IMPLANTATION OF DONOR BURSA
MATERIALS AND METHODS

The fertile eggs and the chickens used throughout this study were from the regional, random-bred White Leghorn population obtained from the Department of Poultry Science of The Ohio State University. Complete details of each of the procedures have been described in the appendixes and will be briefly described here.

**Bursectomy**

The fertile eggs were divided into two groups: (A) One group of eggs (experimental) was given a 0.1 ml. (2.5 mg.) injection of testosterone propionate in sesame oil (Schering) on the fifth day of incubation. Administration of testosterone or its derivatives on the fifth day of incubation causes complete inhibition of the bursa of Fabricius (23, 38, 40, 48, 57) with no chance for proliferation of lymphocytes or stromal cells (1, 2, 3). (B) A second group of eggs, not treated with testosterone, served as a source of normal bursas for subsequent implantation as well as controls, i.e., animals with intact bursas and having normal immunological reactivity. All chickens were hatched and maintained under the standard conditions and diet as employed by the Department of Poultry Science.
Implantation of bursa and immunization

The bursas used for implantation were obtained from chicks seven days after hatching. Bursas were removed surgically, placed in Hank's Balanced Salt Solution, and divided into four equal pieces. A piece of bursa was surgically implanted either subcutaneously or intraperitoneally into one group of seven-day-old, testosterone-bursectomized chicks. Another group of seven-day-old, testosterone-bursectomized chicks was surgically implanted subcutaneously or intraperitoneally with a bursa enclosed within a Millipore diffusion chamber. Millipore diffusion chambers were constructed of two 25 mm. plastic cellulose filters of 0.45 micra porosity (Millipore Filter Company, Bedford, Massachusetts) and sealed with MF Cement No. 2. In comparative studies, filters of a smaller porosity, 0.1 micra, were used. As a control, hormonally bursectomized chicks were implanted with empty diffusion chambers (Figure 1).

On the ninth day after hatching, i.e., one day following the surgical procedures, chicks from the control and experimental groups were given a 1.0 ml. intramuscular injection of killed Salmonella typhimurium, standardized at $3 \times 10^9$ cells/ml. Four weeks later, a second injection of S. typhimurium was given. Two weeks after the second injection of antigen, spleens were removed for histological examination.
Histological preparation and examination

At the termination of the experimental procedure, i.e., seven weeks, specimens of spleen from control intact chickens, hormonally bursectomized chickens implanted with bursa alone or bursa-filled diffusion chambers and hormonally bursectomized chickens implanted with empty diffusion chambers were studied. Specimens of spleen were removed from killed chickens and fixed in formal-sublimate-acetic acid, embedded in paraffin, sectioned at 5 micra and stained with 0.1 percent aqueous toluidine blue.

Cross sections of spleen of comparable size from all groups were randomly selected and examined microscopically for the presence and number of lymphoid nodules. The number of lymphoid nodules per section have been expressed as arithmetic means.

Cytological analysis of representative areas of red pulp from the spleens of both the control and experimental groups was obtained by microscopic examination under oil immersion. Representative areas of red pulp chosen subjectively on the basis of similar cellular density under low magnification. The criteria for the determination of cell types were: (A) "blast" cells, large round cells with deeply basophilic cytoplasm and a round centrally located nucleus containing one or two nucleoli (e.g., see Figure 15), (B) plasma cells, smaller oval cells with basophilic cytoplasm containing a lightly stained Golgi region and round,
eccentrically-placed, hyperchromatic nucleus (e.g., see Figure 7), (C) intermediate cells, cells which were intermediate in size between the typical "blast" cells and the typical plasma cells. The classification of the intermediate cells, whether of lymphocytic or plasmacytic nature, could not be determined precisely. Small and medium lymphocytes and reticular cells which were abundant in the representative areas were not considered in the cytological analysis.
RESULTS

Histological structure of the spleen: The control chicken

The chicken spleen consisted of red and white pulp. The distinction between these two types of lymphocytic tissue was found to be less sharp in the chicken than in the mammal. The white pulp consisted of dense non-nodular accumulations of predominantly small lymphocytes which surrounded small arteries and arterioles. The lymphocytic masses of white pulp were less pronounced than those seen in the white pulp of the mammalian spleen. The red pulp consisted of loose lymphocytic tissue which was in large part occupied by the so-called Schweigger-Seidel sheaths (sheathed arteries). The Schweigger-Seidel sheaths are oval or spindle shaped thickenings of arteriole branches usually surrounded by small lymphocytes (Figure 4). The striking feature of the spleen of the chicken was the occurrence of circular or oval aggregations of tightly grouped lymphocytic cells, i.e., lymphocytic nodules (Figures 4–7). The lymphocytic nodules were completely encapsulated by a thin fibrous membrane and were usually found in juxtaposition to a small artery (Figures 4 and 6). "Blast" cells, large and medium lymphocytes, and reticular cells were found in the nodule. Plasma cells were
Figure 4

Two lymphocytic nodules (ln) adjacent to a single artery (a) with a Schweigger-Seidel sheath (ss) in the red pulp from the spleen of a control chicken. Toluidine blue. 153x

Figure 5

Two lymphocytic nodules (ln) from the spleen of a control chicken. Note the fibrous membrane encapsulating the nodules. Toluidine blue. 383x

Figure 6

Single lymphocytic nodule (ln) from the spleen of a control chicken adjacent to an artery (a). Toluidine blue. 288x

Figure 7

Single lymphocytic nodule (ln) from the spleen of a control chicken showing capsular membrane (m) and two plasma cells (p) in the adjacent red pulp. Toluidine blue. 615x
not seen in the lymphocytic nodules, but were found to be in the red pulp. The concentration of plasma cells was found to be relatively high, 6.4 cells/oil immersion field; "blast" cells were also found in abundance, 8.3 cells/oil immersion field. Cytological analyses of red pulp areas in the spleens of control chickens have been summarized in Table 3. Lymphocytic nodules were abundant throughout the sections studied, 15.4 nodules/section (Table 4), and were distributed randomly.

**TABLE 3**

CYTOLOGICAL ANALYSIS OF SELECTED RED PULP AREAS OF THE SPLEENS OF CONTROL, BURSECTOMIZED AND BURSECTOMIZED-BURSA IMPLANTED CHICKENS

<table>
<thead>
<tr>
<th>Group</th>
<th>Oil Immersion Fields Counted</th>
<th>Mean No. of &quot;Blast&quot; Cells</th>
<th>Mean No. of Intermediate Cells</th>
<th>Mean No. of Plasma Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-bursectomized control</td>
<td>9</td>
<td>8.3</td>
<td>6.2</td>
<td>6.4</td>
</tr>
<tr>
<td>Bursectomized with empty diffusion chamber implanted</td>
<td>5</td>
<td>1.8</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>Bursectomized with bursa-filled diffusion chamber implanted</td>
<td>8</td>
<td>6.0</td>
<td>5.0</td>
<td>0.45</td>
</tr>
<tr>
<td>Bursectomized with bursa alone implanted</td>
<td>5</td>
<td>6.4</td>
<td>4.4</td>
<td>0.6</td>
</tr>
</tbody>
</table>

*The variable number of areas counted is due to the lack of similarly diffuse areas of red pulp among the bursectomized groups.*
MEAN NUMBER OF LYMPHOCYTIC NODULES OF THE SPLEENS OF
CONTROL, BURSECTOMIZED AND BURSECTOMIZED-
BURSA IMPLANTED CHICKENS

<table>
<thead>
<tr>
<th>GROUP</th>
<th>No. of Animals Examined</th>
<th>No. of Sections Counted</th>
<th>Mean No. of Follicles/Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-bursectomized control</td>
<td>10</td>
<td>24</td>
<td>15.1</td>
</tr>
<tr>
<td>Bursectomized with empty diffusion chamber implanted</td>
<td>7</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Bursectomized with bursa-filled diffusion chamber implanted</td>
<td>6</td>
<td>17</td>
<td>5.6</td>
</tr>
<tr>
<td>Bursectomized with bursa alone implanted</td>
<td>6</td>
<td>17</td>
<td>5.3</td>
</tr>
</tbody>
</table>

The bursectomized chicken

The spleens of bursectomized chickens implanted with empty diffusion chambers showed a general decrease in lymphocyte concentration of the red pulp associated with a relative increase in the number of S-S sheaths (Figures 8 and 9). No lymphocytic nodules were found in the spleens of the bursectomized chickens. There were dense, non-nodular aggregations of small and medium lymphocytes corresponding to the white pulp of the spleen of the control chicken, however, the cells were more massed than in the control (Figures 8-11). No fibrous membrane capsule was found to exist around the lymphocytic aggregations. Plasma cells were lacking in the areas of red pulp studied in the spleen of the
Figure 8
Spleen from bursectomized chicken showing diffuse lymphocytic area (dl) corresponding to the white pulp. Note the decreased cellular concentrations of the red pulp and the increased number of Schweigger-Seidel sheaths (ss). Toluidine blue. 104x

Figure 9
Spleen from bursectomized chicken showing diffuse lymphocytic area (dl) and Schweigger-Seidel sheaths (ss). Toluidine blue. 104x

Figure 10
Spleen from bursectomized chicken with diffuse lymphocytic area (dl) and adjacent artery (a). Toluidine blue. 153x

Figure 11
Spleen from bursectomized chicken showing lymphocytic area (dl) surrounding an artery. Toluidine blue. 153x
bursectomized chicken. "Blast" cells were reduced in number, 1.8 cells/oil immersion field, as were intermediate cells, 1.6 cells/oil immersion field, in the areas of red pulp studied. Small lymphocytes were found to be the most predominate cells in the areas studied. Cytological analyses of the red pulp of the spleens of bursectomized chickens implanted with empty diffusion chambers have been summarized in Table 4.

The bursectomized chicken implanted with bursa alone or bursa-filled diffusion chambers

Lymphocytic nodules were found to be present in the spleens of bursectomized chickens implanted with bursa alone (Figure 12), and bursectomized chickens implanted with bursa-filled diffusion chambers (Figures 13-15). The shape of the nodule was less uniform than found in the control chicken. Some nodules were small with a relatively thick, fibrous capsule (Figure 14), while other nodules were large with an incomplete capsule (Figures 12 and 13). The number of nodules per spleen section was fewer in the bursectomized-implanted chickens (bursa alone implanted, 5.3 nodules/section; bursa-filled diffusion chamber implanted 5.6 nodules/section) than in the control chickens (15.1 nodules/section). Plasma cells were observed rarely in the red pulp areas of the bursectomized chickens implanted with bursa. The concentration of lymphocytic nodules and the cytological analyses of red pulp areas
in the spleens of bursectomized chickens implanted with bursa alone or bursa-filled diffusion chambers have been summarized in Tables 3 and 4.
Figure 12

Single lymphocytic nodule (ln) associated with artery (a) from spleen of bursectomized chicken implanted with bursa alone. Note that the membrane (m) is discontinuous (arrow). Toluidine blue. 288x

Figure 13

Single lymphocytic nodule (ln) from spleen of bursectomized chicken implanted with a bursa-filled diffusion chamber. Note that membrane (m) is not continuous (arrow). Toluidine blue. 288x

Figure 14

Single lymphocytic nodule (ln) from the spleen of a bursectomized chicken implanted with bursa-filled diffusion chamber. Note the thickness of the membrane (m). Toluidine blue. 383x

Figure 15

Single lymphocytic nodule from spleen of bursectomized chicken implanted with bursa-filled diffusion chamber showing "blast" cell (b) in the vascular channel adjacent to capsule of the nodule. Toluidine blue. 615x
DISCUSSION

Lymphocytic nodules shown to be absent in spleens of burssectomized chickens have been found to be present following implantation of bursa (either bursa alone or a bursa-filled diffusion chamber). By contrast, plasma cells also absent in the spleens of burssectomized chickens were not stimulated to form in the spleens of burssectomized chickens following implantation of bursa. Evidence that the mechanism of development of the lymphocytic nodules was by means of a non-cellular, humoral substance was strongly suggested by the finding that bursa enclosed within a cell-impermeable Millipore diffusion chamber was capable of forming the lymphocytic nodules. The observation that in ovo the testosterone burssectomy resulted in an absence of the lymphocytic nodules and plasma cells is in agreement with the findings of Cooper et al. (17) These authors (17) suggest that the lymphocytic nodules and the plasma cells were dependent upon the bursa of Fabricius for their development and function in the spleens of chickens. Such findings led them to coin the terms "bursa-dependent follicles" for the lymphocytic nodules and "bursa-dependent system" for the nodules and the plasma cells.
Lymphocytic nodules are not present in the spleens of chickens at hatching, but are recognized morphologically by four to five weeks of age (17). Lymphocytic nodules have been observed to increase in size in the spleens of chickens with visceral lymphomatosis (34) and to increase in number following infection with *Plasmodium gallinaceum* (54). The increased size and number of the nodules has been regarded as pathological, although smaller and less numerous nodules have been observed in the spleens of normal chickens. The function of the lymphocytic nodule (bursa-dependent follicle) has not been explained. It is apparent that the increased number and size of the lymphocytic nodules found in certain pathological conditions (34, 54) is associated with immune reactions. Studies which observed the histopathological changes in the spleens of chickens infected with malaria (54) indicated that not only were there increased numbers and size of the lymphocytic nodules, but cytological changes took place within the nodule, i.e., increased mitosis and an increase in the number of "blast" cells, and large and medium lymphocytes.

Studies of the spleens of normal chickens following antigenic stimulation (35, 36) showed increased numbers of "blast" cells and plasma cells present. Such studies suggested strongly that plasma cells were involved in the synthesis of antibody in the chicken. The observation that the plasma cell was responsible for the production of
antibody has been substantiated by many investigators (18, 19, 24, 41, 52), and direct observation by means of the fluorescent antibody technique has localized antibody in the cytoplasm of plasma cells (16, 42, 52, 62). In the present study no plasma cells were observed in the lymphocytic nodule of the control chicken, but were present in the red pulp of the spleen. The bursectomized chickens implanted with bursa (either bursa alone or a bursa-filled diffusion chamber) were found to be capable of producing antibody (bursa alone, mean titer 1:225; bursa-filled diffusion chamber, mean titer 1:105), although plasma cells were rarely observed in the red pulp. Two alternatives exist for the production of antibody in the bursectomized-implanted chickens in the absence of plasma cells: (1) the production of antibody by lymphocytic tissue in other regions of the body, viz., lower cecum and the subcutaneous region of the neck, and (2) the production of antibody by the lymphocytic nodules. The possible alternative of antibody production by accessory lymphocytic tissue is unlikely since in ovo testosterone bursectomy apparently eliminates these areas (23).

The lymphocytic nodules (bursa-dependent follicles) of the chicken spleen bear a striking resemblance to the secondary or germinal centers of the mammalian lymph nodes and spleen. The reaction of the lymphocytic nodules of the chicken spleen to infection appears to be similar to that of the germinal centers in mammals under the same condition,
viz., increased size and number of nodules and an increased mitotic activity and cellular proliferation (54). Antibody has been detected in the germinal centers of the lymphocytic nodules of mammalian lymph nodes and spleen using the fluorescent antibody technique (16, 62), as well as in the discrete lymphocytic nodules of the spleen of the chicken (62). The majority of the cells within the lymphocytic nodule exhibited demonstrable antibody. The plasma cells present in the red pulp also contained demonstrable antibody with the fluorescent-antibody technique. Our preliminary attempts to demonstrate antibody in the lymphocytic nodules and plasma cells have proven unsuccessful. The reason for this failure, though not apparent, would seem to reside in the many pitfalls of the fluorescent-antibody procedure.

The failure of the implants of bursa to stimulate the formation of plasma cells in the spleens of bursectomized chickens may be the explanation for the decreased antibody production by these birds when compared with control chickens (Part I). In similar studies (27, 28) it was found that implantation of bursa alone, although having no effect upon the formation of lymphocytic nodules, greatly increased the number of plasma cells. Lymphocytes from the implanted bursa of Fabricius were reportedly (27, 28) viable for eight to ten days after implantation. The present study indicated that lymphocytic nodules (bursa-dependent follicles) could be reconstituted by a non-cellular substance capable of passing
through a cell-impermeable Millipore diffusion chamber. It is possible that immunologically competent lymphocytes migrate from the bursa to the spleen, there to transform into plasma cells under the stimulus of an antigen. The cellular origin of the plasma cell remains unresolved; however, most investigators feel that lymphocytes transfer into plasma cells in areas where increased concentrations of plasma proteins generally occur, e.g., lymph nodes, spleen and other highly vascular organs (30, 42, 52).

Further investigations are planned to study the formation and development of the lymphocytic nodules (bursa-dependent follicles) of the chicken spleen under normal and immunologically reactive conditions utilizing the fluorescent-antibody technique.
SUMMARY

The bursa of Fabricius, a lymphoepithelial organ, peculiar to birds has been found to be essential for the production of antibody in the chicken. The bursa is not the site for the production of antibody, but acts in an indirect role to establish the potential to form antibody. The spleen of the chicken is the organ responsible for the formation and elaboration of antibody. The relationship of the bursa of Fabricius to the spleen has been studied in the White Leghorn chicken. The results show:

1. The spleen of the intact control chicken immunized with Salmonella typhimurium contained discrete lymphocytic nodules and plasma cells.

2. The spleen of the chicken bursectomized in ovo by an injection of testosterone at the fifth day of incubation showed complete absence of lymphocytic nodules and plasma cells.

3. The spleens of chickens bursectomized in ovo by testosterone injection at the fifth day of incubation and implanted with donor bursa alone or donor bursa enclosed within cell-impermeable Millipore diffusion chambers showed discrete lymphocytic nodules. Plasma cells, however, were observed rarely.
The evidence presented suggests that the bursa of Fabricius is responsible for the development of the lymphocytic nodules in the spleen of the chicken. The evidence strongly suggests that the bursa elaborates a non-cellular (hormone-like) substance which re-establishes the lymphocytic nodules in the spleens of bursectomized chicks. The suggestion is made that the presence of plasma cells in the spleen of the chicken may require transmigration of lymphocytes from the bursa. The role of the lymphocytic nodules and the plasma cells in antibody production is discussed.
PART III

TRANSFER OF IMMUNOLOGICAL REACTIVITY
MATERIALS AND METHODS

The fertile eggs and the chickens used throughout this study were from the regional, random-bred White Leghorn population obtained from the Department of Poultry Science of The Ohio State University. Complete details of each of the procedures have been described in the appendixes and will be briefly described here.

**Bursectomy**

The fertile eggs were divided into two groups: (A) One group of eggs (experimental) was given a 0.1 ml. (2.5 mg.) injection of testosterone propionate in sesame oil (Schering) on the fifth day of incubation. Administration of testosterone or its derivatives on the fifth day of incubation causes complete inhibition of the bursa of Fabricius (23, 38, 40, 48, 57) with no possible chance for proliferation of lymphocytes or stromal cells (1, 2, 3). (B) A second group of eggs, not injected with testosterone, served as a source of chickens having normal immunological reactivity. All chickens were hatched and maintained under the standard conditions and diet as employed by the Department of Poultry Science.
Implantation of lymphocytic organs and immunization

Lymphocytic organs used for implantation, viz., thymus, spleen and bursa of Fabricius were removed seven weeks after hatching from chickens which had been injected twice with the antigen (Salmonella typhimurium). The seven-week-old, immunized birds were known to have a mean antibody titer of 1:330 to S. typhimurium. The lymphoid organs were surgically removed and placed in Hank's Balanced Salt Solution. Following a wash in balanced salt solution, the organs were cut into pieces approximately 10 mm. square and enclosed within a Millipore diffusion chamber. Millipore diffusion chambers were constructed of two 25 mm. plastic cellulose filters of 0.45 micra porosity (Millipore Filter Company, Bedford, Massachusetts) and sealed with MF Cement Formulation No. 2.

Hormonally bursectomized chicks seven days old served as recipients. The bursectomized recipients were divided into three groups: (1) one group received a graft of lymphoid organ enclosed within a Millipore diffusion chamber combined with a 1.0 ml. injection of killed S. typhimurium (standardized at 3 x 10^9 cells/ml.); (2) a second group received an organ implant and no injection of antigen; and (3) a third group received no organ implant, but received a 1.0 ml. intramuscular injection of S. typhimurium. The Millipore diffusion chambers containing lymphocytic organs were implanted subcutaneously in the bursectomized chicks. Control
intact chicks seven days old received an intramuscular injection of 1.0 ml. *S. typhimurium*. All birds were maintained under the standard conditions and diet as employed by the Department of Poultry Science.

**Agglutination tests**

Both control and experimental animals were maintained until the seventh week. At the seventh week blood samples were obtained by cardiac puncture, placed in centrifuge tubes and allowed to clot at room temperature. The serum was removed from the clot and stored at 4°C after centrifugation. Agglutination tests were made by adding 0.25 ml. of the standard antigen to 0.25 ml. of the serum in serial dilution from 1:2 to 1:1024. Tubes were incubated at 45°C for two hours then refrigerated for 24 hours and antibody titers determined. Agglutination above a titer of 1:4 was considered positive, i.e., indicating the presence of antibody to *S. typhimurium*. Agglutination was graded subjectively from 4+ to 0. The end point of antibody activity was taken as the last tube showing a 1+ agglutination. The antibody titers have been expressed as arithmetic means.
RESULTS

Antibody production in control and bursectomized chickens following a single injection of antigen

Following a single injection of the antigen, *S. typhimurium*, at seven days of age normal, intact, control chickens had a positive antibody production in 20 of 25 birds when tested at seven weeks of age. The mean antibody titer was 1:35. By direct contrast none (10 of 10) of the bursectomized birds were able to produce demonstrable antibody to *S. typhimurium*. The results of antibody production in normal and bursectomized chickens have been summarized in Table 3 and Figure 16.

Antibody production in bursectomized chickens grafted with lymphocytic organs

Thymus. The bursectomized chicks which received an implanted graft of thymus followed by an injection of *S. typhimurium* were unable to produce antibody in 20 of 20 birds. No demonstrable production of antibody was found in 15 of 15 bursectomized chickens implanted with a graft of thymus without subsequent injection of antigen.
Figure 16

Mean titers of antibody production in (1) hormonally bursectomized chickens implanted with bursa followed by injection of antigen; (2) non-bursectomized control chicks injected with antigen; (3) hormonally bursectomized chicks implanted with spleen followed by injection of antigen; and (4) hormonally bursectomized chicks not implanted, but injected with antigen.
Bursa. A positive production of antibody was obtained from 24 of 27 birds having both an implanted graft of bursa and an injection of antigen. A mean titer of antibody of 1:16 was obtained. Implantation of a graft of bursa without injection of *S. typhimurium* failed to reconstitute production of antibody in 21 of 21 birds.

Spleen. Bursectomized chickens implanted with a graft of spleen followed by an injection of antigen produced antibody in 28 of 32 birds. The mean antibody production was 1:132. Implantation of a graft of spleen without an injection of *S. typhimurium* failed to result in the production of antibody in 22 of 22 implanted chickens.

The scheme of organ transfer has been summarized in Table 5.

**TABLE 5**

SCHEME OF LYMPHOCYTIC ORGAN TRANSFER TO BURSECTOMIZED CHICKENS

<table>
<thead>
<tr>
<th>Donor Tissue</th>
<th>Recipient</th>
<th>Antigen Injected</th>
<th>Mean Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymus</td>
<td>Bursectomized 7-day-old chick</td>
<td><em>S. typhimurium</em></td>
<td>0</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>Hormonally</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Secondarily Immunized 7-week-old Chicken</td>
<td>Bursa of Fabricius</td>
<td>Hormonally</td>
<td>1:16</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>Bursectomized 1-day-old chick</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td>Bursectomized 7-day-old chick</td>
<td><em>S. typhimurium</em></td>
<td>1:132</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>Hormonally</td>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>
The results of antibody production in chickens implanted with grafts of lymphoid organs have been summarized in Table 6 and Figure 16.

### TABLE 6

ANTIBODY PRODUCTION IN BURSECTOMIZED AND CONTROL CHICKENS, AND THE EFFECT OF LYMPHOCYTIC ORGAN TRANSPLANT ON ANTIBODY-PRODUCING DEFICIENCY IN BURSECTOMIZED CHICKENS

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Animals</th>
<th>Type of Implant</th>
<th>Injection of Antigen</th>
<th>Mean Antibody Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Chicken</td>
<td>20/25</td>
<td>none</td>
<td>+</td>
<td>1:35</td>
</tr>
<tr>
<td>Bursectomized Chicken</td>
<td>15/15</td>
<td>thymus</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bursectomized Chicken</td>
<td>20/20</td>
<td>thymus</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Bursectomized Chicken</td>
<td>21/21</td>
<td>bursa</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bursectomized Chicken</td>
<td>24/27</td>
<td>bursa</td>
<td>+</td>
<td>1:16</td>
</tr>
<tr>
<td>Bursectomized Chicken</td>
<td>22/22</td>
<td>spleen</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bursectomized Chicken</td>
<td>28/32</td>
<td>spleen</td>
<td>+</td>
<td>1:132</td>
</tr>
<tr>
<td>Bursectomized Chicken</td>
<td>18/18</td>
<td>none</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>
DISCUSSION

The transfer of immunologically competent cells to an immunologically deficient recipient has been found to be a useful technique in the examination of the antibody-producing capacity of the transferred donor cells (14, 15, 43, 44, 56). Circulating antibody may be found in the recipient if antigenic stimulation is given to the recipient after the transfer of immunologically competent cells (14, 15). In the present study lymphocytic organs from immunologically reactive chickens, those which showed a high titer of antibody to *S. typhimurium* were implanted into chickens which were immunologically deficient, i.e., hormonally bursectomized birds which had no prior antigenic stimulation. This study indicated that spleens from immunologically reactive donor chickens were capable of transferring immunological reactivity to immunologically deficient chicks. The amount of antibody produced by hormonally bursectomized recipients is greater than that produced by the control chickens following a single injection of antigen. This finding suggests that the donor spleen produced a secondary or anamnestic response in the recipient.

The intact control chickens showed positive antibody production to a single injection of antigen (1:35), while the
hormonally bursectomized chickens not implanted with donor lymphocytic organs, but injected with antigen were completely unable to produce demonstrable antibody. This finding is in agreement with other studies of the antibody-producing capacity of bursectomized chickens (12, 20, 21, 22, 23, 26, 27, 28, 38, 47).

The thymus had no ability to induce antibody production in the bursectomized chicken when implanted either with or without subsequent antigenic stimulation. This finding may be accounted for by the concept of a dissociation of immune response in the chicken (53, 59, 61). It has been proposed that in the chicken there are at least two functionally different populations of immunologically competent cells, the development of which is controlled by the bursa of Fabricius and the thymus. The thymus is thought to be primarily concerned with the direct cellular response to tissue grafts, i.e., homograft rejection, while the bursa is associated with antibody formation.

Implantation of the bursa of Fabricius into hormonally bursectomized chickens without antigenic stimulus produced no demonstrable antibody. However, when the implantation was combined with an injection of S. typhimurium, a mean titer of 1:16 was obtained. The mean titer of antibody produced by the bursectomized chickens implanted with bursa was somewhat lower (1:16), than the mean titer produced by the intact control chicken (1:35) given a single
injection. It is felt that if the cells of the implanted bursa were capable of producing antibody, the multiple antigenic stimulation, i.e., twice in the donor and once in the recipient should have produced a mean titer of antibody in the hormonally bursectomized recipient greater than in the control chicken. The bursa is, therefore, not a site for the production of antibody, but has been found to elaborate a non-cellular humoral substance which reconstitutes the potential for the production of antibody in bursectomized chickens (50).

There is evidence that the spleen is associated with antibody formation, since splenectomy reduces the immune response of chickens (13, 17, 35, 36, 43, 49, 55, 63). The spleen is considered to be the site of antibody-producing cells (plasma cells) in the chicken (35, 36). In the present study no antibody production was obtained from recipients if donor spleens were implanted without subsequent injection of antigen. If the injection of antigen was combined with spleen implantation, a relatively high antibody titer (1:132) was obtained. This is in agreement with the immune transfer studies by Trnka (56) who found that either injection of spleen cells from adult chickens alone or injection of antigen alone produced no antibody in the recipient. It would appear that the immunologically competent cells of the spleen must come in contact with the antigen before antibody formation can take place in the recipient. Evidence that the
donor spleen induces a secondary or anamnestic response in
the recipient is strongly suggested by the finding that bur­
sectomized chickens implanted with spleens from donors in­
jected with antigen twice had higher antibody titers than
normal control chickens after one injection. The secondary or
anamnestic response is associated with a rapid rise in anti­
body titer following re-immunization (11). This observation
is in agreement with studies on mammalian spleen cell transfer
in which spleen cells stimulated with antigen in vitro pro­
duced a secondary response in recipients (43, 44).

Attempts at transfer of implanted organs from primary
recipients to secondary recipients failed to produce de­
monstrable antibody in the implanted animal.

From the experimental evidence presented in this
study it was not possible to determine whether the circu­
lating antibody present in the immunologically inert recipi­
ents of spleens from donor animals was produced by the
implanted donor spleen or by the spleen of the recipient.
Due to the surprising results of this study this aspect was
overlooked until the writing of this dissertation.

Two experimental procedures should be performed which
would give evidence indicating the site of production of anti­
body: (1) Evidence that the implanted donor spleen was the
site of production of antibody would be established if circu­
lating antibody were found in chicks neonatally splenectomized
prior to implantation; (2) Evidence indicating that the spleen of the
recipient is the site of production would be established if implantation of donor spleen reconstituted the bursa-dependent system in the spleen of the recipient.

Plans are now underway in our laboratory to repeat the present study employing these experimental procedures.
SUMMARY

The transfer of lymphocytic organs from immunologically reactive donor chickens to immunologically inert recipients tests the capacity of the transferred organ to produce antibody in the recipient. The production of antibody to *S. typhimurium* has been studied in the hormonally bursectomized White Leghorn chicken grafted with either thymus, bursa of Fabricius or spleen from donor adult birds producing a high titer of antibody to *S. typhimurium*. The results show:

1. Implantation of donor thymus failed to transfer the production of antibody to bursectomized chickens.

2. The bursa of Fabricius induced production of antibody in concentrations lower than the normal intact bird. The bursa does not function as a site for the production of antibody, but initiates antibody production by means of a non-cellular substance.

3. Implantation of donor spleen resulted in the production of antibody in the recipient greater than in the intact, control chicken.

The evidence presented adds support to the view that the spleen is the organ responsible for production of antibody in the chicken. Multiple antigenic stimulation of the
donor spleen in vivo produces an anamnestic response in the recipient following a single injection of antigen.

Experimental procedures have been suggested to ascertain whether the implanted spleen or the spleen of the implanted recipient is responsible for the production of antibody in bursectomized chickens implanted with donor spleens.
GENERAL SUMMARY

The bursa of Fabricius and its role in the immune response in chickens has been studied in this investigation. The results obtained show:

1. Chickens hormonally bursectomized by an injection of testosterone on the fifth day of incubation were unable to form antibody to *Salmonella typhimurium*.

2. Chickens hormonally bursectomized by an injection of testosterone on the fifth day of incubation and subsequently implanted with bursa (either bursa alone or a bursa-filled diffusion chamber) showed a reconstituted antibody production.

3. The spleens of intact control chickens producing a high titer of antibody to *S. typhimurium* had discrete lymphocytic nodules similar to secondary or germinal centers of mammalian lymph nodes and spleens.

4. The spleens of chickens hormonally bursectomized by testosterone injection on the fifth day of incubation were found to be lacking the lymphocytic nodes.

5. The spleens of chickens bursectomized by testosterone injection on the fifth day of incubation and implanted with bursa (either bursa alone or a bursa-filled diffusion chamber) showed the lymphocytic nodules.
6. Implantation of thymus from donor chickens producing a high titer of antibody to *S. typhimurium* failed to transfer the production of antibody to hormonally bursectomized chickens.

7. Implantation of spleen from donor chickens producing a high titer of antibody to *S. typhimurium* resulted in the production of antibody in hormonally bursectomized recipients greater than the intact control chicken following a single injection of antigen.

The results obtained in this dissertation have provided additional support for the view that the bursa of Fabricius plays an indirect, but absolutely essential role for the capacity to form antibody in the chicken. The evidence presented suggests that the bursa of Fabricius elaborates a non-cellular, hormone-like, substance which reconstitutes antibody production in bursectomized chickens. The humoral factor from the bursa also enhances development of the discrete lymphocytic nodules (bursa-dependent follicles) in the spleens of bursectomized chickens. Transplantation of spleen from donor chickens stimulated secondarily with antigen, produces an anamnestic response in recipients rendered immunologically inert by testosterone bursectomy following single injection of antigen.
APPENDIXES
APPENDIX A

METHOD OF TESTOSTERONE BURSECTOMY

The fertile eggs obtained from the Department of Poultry Science were gathered fresh and set in the incubator. All eggs were incubated with the air cell upward in the incubator trays. Time of incubation was begun 24 hours after the fertile eggs were set, i.e., day one. On the fifth day of incubation all eggs were removed from the incubator and the shell area over the air cell (the more pointed portion) was cleansed with 70 per cent alcohol. A small hole was punched in the shell using an egg punch obtained at the Department of Poultry Science. A 20 gauge needle was placed in the hole and passed diagonally through the air cell into the albumin. The need for the diagonal direction is to insure against direct injury to the developing embryo. By means of steady, even depression of the syringe plunger 0.1 ml. (2.5 mg.) of testosterone propionate (Schering) is injected into the albumin. A rapid or uneven depression of the plunger may result in injury to the embryo or extrusion of the egg contents into the air cell, either of which reduce viability. After injection, the needle was carefully removed and the hole sealed with melted "Paraplast" or paraffin
painted over the hole. After the hole was sealed and all eggs in the incubator trays had been injected, the trays were returned to the incubator. Injection time for any given tray should be kept to less than 30 minutes to reduce the chance of retarded development and reduction of viability. Room temperature should be between 70° to 75° C. to eliminate undo cooling of the eggs during the injection procedure.
APPENDIX B

METHOD OF DIFFUSION CHAMBER CONSTRUCTION

The organs to be implanted were removed surgically from the donor animal and placed in Hank's Balanced Salt Solution (any other medium capable of preserving the viability of tissues is also suitable, e.g., Difco T. C. 199, Difco Laboratories, Detroit, Michigan. After a wash in this medium, the organs were cut into suitable pieces, i.e., no larger than 10 mm. square. Any piece larger than 10 mm. square lowers the tissue volume to diffusion area ratio and results in reduced viability of the tissue (Ref. Dr. Clinton A. Olmsted, Head, Cell Biology Division, Institute for Lipid Research, Berkeley, California). A single organ piece was removed from the wash medium and placed on a Millipore filter disc 25 mm. in diameter with a pore size of 0.45 micra or 0.1 micra. A second Millipore filter was placed over the tissue and the edges of the two discs were sealed with MF Cement No. 2 (specific for Millipore filter to Millipore filter bonding) applied with a wooden applicator stick. All Millipore filter discs and MF cement were obtained from the Millipore Filter Corp., Bedford, Mass. The edges of the chamber must be sealed with great care to insure against
leaks. Until proficiency is gained in sealing of the chambers it is suggested that all chambers be checked for leaks with a dissecting microscope. After the chambers were sealed, they were placed again in the wash medium and allowed to sink, this assured that the tissue was bathed in the medium. The chambers could be left for up to one hour in the wash medium. A longer duration was felt to reduce the viability of the tissue although no specific experimental evidence was collected to indicate this fact.
APPENDIX C

METHOD OF IMPLANTATION OF DIFFUSION CHAMBERS

Animals for implantation were ether-anesthetized and placed on their dorsal aspect on the dissecting table with the feet toward the operator. A small patch of ventral down (approximately 3 cm. square) was removed by gentle plucking (vigorous plucking tends to tear the skin). The surgical area is prepared with 70 per cent alcohol and a 3 cm. incision is made caudal to the keel. Scissors were found to be better for the incision than a scalpel since no underlying muscle layers were incised. If subcutaneous implantation was employed a probe was introduced into the incision to bluntly dissect the skin from the fascia overlying the muscle layers. A previously prepared diffusion chamber was placed under the skin and maneuvered to a position adjacent to the brachial wing vein (Ref. Meuller et al., p. 371 (39)). The incision was closed by three 9 mm. autoclips (Clay-Adams, Inc., N. Y.) and the chick was allowed to recover from the affects of the anesthetic before returning it to its pen.

If intraperitoneal implantation was employed, after the initial skin incision the abdominal wall musculature was
carefully incised in midline caudal to the keel using a scalpel and the peritoneal cavity opened. A previously constructed diffusion chamber was placed in the peritoneal cavity dorsal to the keel. No specific closure of the ventral wall musculature was used since it closed adequately when the skin incision was closed by three 9 mm. autoclips. Again the chick was allowed to recover from the effects of the anesthetic before returning it to its pen.

Care must be exercised in the administration of anesthetic as this proved to be the only source of surgical mortality. Using ether as an anesthetic two minutes time in a closed anesthetizing chamber, 5" in height and 3" in diameter was found to provide adequate anesthesia for a bursectomized chick seven days old.
APPENDIX D

PREPARATION OF ANTIGEN

Two test tube slant cultures of standard nutrient agar medium were inoculated with a stock culture of *Salmonella typhimurium* (Department of Microbiology, The Ohio State University). The slant cultures were incubated in an oven at 37° for 18 hours and transferred to 5 or 6 Petri dish cultures of standard nutrient agar. The transfer was accomplished by adding 2.0 ml. of normal bacterial growth gently with a sterile wire loop. The entire surface of the agar of each Petri dish culture was heavily innoculated with the bacterial suspension. After inoculation the Petri dish cultures were incubated in an oven at 37° C. for 18 hours. At the end of the incubation, 5 ml. of 0.6 per cent formal-saline (0.6 ml. formaldehyde to 100 cc. normal saline, ph 7.4) was pipetted onto the surface of the culture and the bacterial growth loosened with a sterile wire loop. The resulting bacterial suspension was transferred to a sterile test tube. Standardization of the bacterial suspension was accomplished by subjectively comparing the suspension with a known standard of $3 \times 10^9$ cells/ml. Formal-saline was added to the bacterial suspension until the turbidity matched that
of the standard. In order to prevent contamination it was found best to freeze the antigen until the day it was used.
APPENDIX E

METHOD OF CARDIAC PUNCTURE

The following method was employed for cardiac puncture of chickens seven weeks of age. A chicken was killed by breaking the neck with a sharp pull of the head while the feet and legs were immobilized. After the reflex spasms ceased, the bird was placed on its dorsal aspect, positioned with the head facing the operator. The thoracic inlet, i.e., the triangular space between the base of the neck and keel, was palpated. An 18 gauge needle attached to a 20 cc. syringe was passed into the inlet in a left diagonal direction. With slight negative pressure on the syringe the heart was probed. Once the heart was punctured, slow, steady retraction of the plunger prevented slipping of the needle from the punctured area. Approximately 10 ml. of blood was removed from each chicken.

In transferring blood from the syringe to a 15 ml. centrifuge tube, it was important to remove the needle and to transfer with as little force as possible to prevent hemolysis of the blood. Marked hemolysis tended to cloud later serological tests.
APPENDIX F

METHOD OF AGGLUTINATION DETERMINATIONS

Agglutination tests were begun by setting up ten agglutination tubes (Test tubes 75 x 12 mm., A. H. Thomas Co., Philadelphia, Pa.). To all ten tubes 0.25 ml. of saline (Phosphate buffered, ph 7.4) was added. To tube one 0.25 ml. of the test antiserum to S. typhimurium (from the blood of the experimental animal) was added and carefully mixed by aspirating most of the mixture into a 1.0 ml. pipette and gently blowing it out five or six times. This tube contained a 1:2 dilution of serum. With the same pipette, 0.25 ml. was transferred to tube two (1:4 dilution), mixed as before and 0.25 ml. transferred to tube three (1:8). This transfer and mix procedure was continued to tube nine from which the 0.25 ml. mixture was discarded thus leaving tube ten as a control. The standard antigen (Salmonella typhimurium) was pipetted to all tubes in 0.25 ml. increments. All tubes were shaken vigorously and incubated in a serological water bath (Chicago Surgical and Electrical Company, Chicago, Ill.) at 45° C. for two hours. After incubation the tubes were refrigerated for 24 hours and agglutination determinations made.
A good light source and a dark background were found desirable for "reading" agglutination tests. Each tube was examined first without shaking; then shaking was applied in order to suspend the sediment. Grading was subjectively determined by the turbidity of each tube. A 4+ reaction was one in which all bacterial cells were clumped at the bottom of the tube and would resuspend as definite granules or flakes upon shaking. In a negative (0) reaction there was no clumping and no sediment. Intermediate agglutination was graded 3+, 2+, and 1+. After the first reading the tubes were refrigerated for 24 hours and the readings were repeated. The highest value obtained in each tube was considered the titer. The end point of antibody activity was taken as the last tube of each series of ten to exhibit a 1+ reaction.
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