TOBEN, Howard Ray, 1941-
EXPERIMENTAL IMMUNOSUPPRESSION.
The Ohio State University, Ph.D., 1971
Anatomy

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EXPERIMENTAL IMMUNOSUPPRESSION

DISSERTATION

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

By

Howard Ray Toben, B.S. M.Sc.

* * * * *

The Ohio State University

1971

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ACKNOWLEDGMENTS

The author wishes to thank the following: Dr. Samuel G. Murphy for column chromatography techniques; Dr. John Delphia for providing incubator space; Dr. James King for assistance in microphotographic techniques; Dr. Jeptha Hostetler for techniques of plastic embedding and cutting; Dr. Max D. Cooper for the techniques of immunoabsorbant columns; Dr. Richard Dorn for providing laboratory space for the embryological aspects of this study; Dr. Ronald Bell for his service in the vivarium; Mr. Paul Hurtibise and Mrs. Karen Cost for their assistance in immunological equipment and techniques; and finally Mrs. Judy Patterson for her assistance in the numerous phases of this research.

Special recognition is due for my adviser, Dr. Ronald L. St. Pierre, for his counsel and guidance throughout this experimentation and writing embodied within this dissertation.

A special thanks to my wife, Petra, for her support during the years as a graduate student.
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<td></td>
<td>saline <em>in ovo</em></td>
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INTRODUCTION

In the chicken, the dichotomy of immunological functions performed by the central lymphoid organs, thymus and the bursa of Fabricius, has been well documented (11). Morphological differentiation of the central lymphoid tissues on the basis of fine structure has been shown by Clawson et al. (7) who demonstrated that lymphocytes from the thymus can be distinguished from lymphocytes from the bursa on the basis of ribosomal populations. Thymic lymphocytes contain only patches of single ribosomes while bursal lymphocytes contain homogeneous populations of ribosomal aggregates consisting of 4-6 ribosomes each. Further differentiation of the thymic and bursal lymphocytes has been shown by Forget et al. (10) on the basis of antigenic specificity as demonstrated by immune adherence techniques.

Kincade et al. (20) have shown functional and morphological changes in the humoral response of chickens after treatment with anti-chicken γM globulins in ovo. Alteration in the bursa morphology as well as loss of M and G classes of immunoglobulin were demonstrated in chickens treated with anti-chicken γM globulin in ovo.

Jankovic et al. (18) demonstrated morphological alterations, but no functional deficits, in the bursa of Fabricius after
administration of 10 daily injections of unabsorbed rabbit-anti-chicken bursa lymphocyte globulin beginning on day 7 after hatching and continuing through day 17. Their technique proved to be extremely lethal with none of the injected embryos reaching hatching.

Alterations in the morphology and function of developing organ systems of embryos as a result of organ specific antisera have been attempted. Brent et al. (5) have used antisera directed against basement membrane of yolk sac to cause malformations and growth retardation in developing rat embryos. Levi-Montalcini and Brooker (23), Levi-Montalcini and Angeletti (22) using antisera directed against nerve-growth-factor inhibited the development of the sympathetic nervous system in rats. Berry (3) demonstrated cardiac malformation in vitro due to inhibitory activities of antisera directed against cardiac contractile protein. Nora (20) also produced cardiac anomalies after in vitro injection of antisera against heart tissue.

The present study was designed to demonstrate further the immunologic dichotomy in chickens treated as embryos with antisera directed against bursal and thymic lymphocytes. The report concerns the serologic activity of antibursal and antithymic globulins, and the effect of these immunologic reagents on central lymphoid tissue and the functional deficits resulting from embryonic administration of the anti-lymphocyte globulins.
MATERIALS AND METHODS

Preparation of the antisera

Thymus and bursa were removed aseptically from 8 week old chickens of the regional random-bred white leghorn population (Department of Poultry Science, The Ohio State University). These tissues were suspended in Locke's solution and disrupted in an Omni Mixer (Sorvall Company, New Haven, Connecticut). The resulting cell suspension (1-2x10^9 cells/cc) was filtered through 50 mesh/inch stainless steel screen. Domestic Mallard ducks were injected intramuscularly with 1x10^9 thymic or bursa cells/cc. A second injection of 1x10^9 thymic or bursa cells/cc was given 14 days after the first injection. Seven days after the second injection the ducks were exsanguinated and the sera harvested (21).

Purification of the antisera

Solid immunoabsorbant columns (see Appendix A) were prepared by coupling membrane associated antigens (see Appendix B) isolated by sonic disruption of bursa lymphocytes and thymic lymphocytes (18) to sepharose 2B (Pharmacia, Piscataway, New Jersey) by cyanogen bromide linkage (30). Duck anti-chicken thymus globulin (DATG) was isolated from plasma proteins by passage over the immunoabsorbant column containing membrane associated antigen of thymic lymphocytes.
The isolated DATG was made specific for thymus cells by passage over immunoabsorbant column containing membrane associated antigens of bursal lymphocytes, which removed any cross-reacting and species specific antibodies. Duck anti-chicken bursa globulin (DABG) was isolated and purified in the same manner as DATG. Normal duck gammaglobulin (NDIg) was isolated from whole sera by Na₂SO₄ precipitation and ion exchange chromatography (2) and absorbed with chicken erythrocytes and leukocytes to remove cross-reacting and chicken species specific antibodies. DABG, DATG, and NDIg were dialized against 0.79% saline for 24 hours (19).

Specificity of the anti-globulins was determined by a modification of micro-leuko agglutination technique of Zmiijewski et al. (32). Instead of peripheral blood leukocytes, lymphocytes from the thymus and bursa served as a source of the cell suspension (see Appendix D).

Embryonic injection

Chicken embryos of 5-6 days of incubation from the regional random-bred white leghorn population were divided into 4 groups. Each group was injected with 10 microliters of one of the following solutions: (1) DABG [1.62 O.D. 280/ml], (2) DATG [1.86 O.D. 280/ml], (3) NDIg [2.01 O.D. 280/ml], and (4) 0.79% saline [see Appendix E]. All injections were made into the chorio-allantoic vesicle. The chickens were hatched and raised in a brooder and individual cages and fed on standard broiler feed.
**Immunization methods**

Chickens were tested for humoral antibody function by immunization with *Brucella abortus* (U. S. Department of Agriculture, Ames, Iowa). The four groups were injected intramuscularly with formalin-killed $1 \times 10^9$ *B. abortus* organisms. At 3, 6 and 9 days after immunization, 5 cc of blood was removed from the brachial vein and the serum harvested. Standard tube bacterial agglutination procedures were carried out on the chicken serum (see Appendix F).

Chickens were tested for cell-mediated immune function by means of skin allografts. The chickens were put under a light anaesthesia (methoxyflurane, Pitman-Moore, Fort Washington, Pennsylvania). A section of wattle was removed from a donor chicken, placed in sterile saline and cut into sections of 8-10 mm$^2$. The graft bed was prepared by removing skin from the lateral thoracic wall of the recipient chicken. The skin allograft (wattle) was shaped to fit the graft bed and sutured into place with 4-6 sutures. No protective covering was applied to the graft site. The grafts were examined at 24 hour intervals until rejection occurred. The progress of rejection was monitored using the criteria of Cooper et al. (9).

**Histological examination**

Thymus, bursa and spleen were fixed in formal sublimate acetic acid (FSA), embedded in paraffin, sectioned at 4 microns and stained with hematoxylin and eosin or toluidine blue for histological
examination. Correlation of immunologic function with lymphoid morphology was accomplished by determination of mean number bursal-dependent areas in spleen sections. This technique was carried out by counting the number of bursal-dependent areas in 6 to 8 sections of spleen of comparable size (see Appendix G).

**Statistical methods**

Student's "t" test was utilized in the analysis of humoral antibody function, of skin graft rejection and determination of the mean bursal-dependent areas of spleen sections. Correlation coefficient test was used to analyze the relationship of antibody titer with mean bursal-dependent areas of spleen.
RESULTS

Isolation and Purification of DATG and DABG

The results of isolation and purification of DATG on thymic and bursal immune absorbant columns are shown in Figure 1. Peak A contained all plasma proteins and specific immunoglobulins which failed to react with the thymic immunoabsorbant column (TIC). Peak B contained eluted immunoglobulins from TIC. This mixture of immunoglobulins had specificities for the thymic specific antigen, species specific antigens for chickens and the cross-reactive antigens associated with bursal lymphocytes. The mixture of immunoglobulins contained within Peak B was placed on the bursal immunoabsorbant column (BIC). Peak C (TIC) represented immunoglobulins having no affinity for BIC, i.e., thymic specific antibodies. The eluted peak D from BIC contained the cross-reacting antibodies to bursal lymphocytes and the species specific antibody for chickens. Peak C was analyzed for thymic specificity by lymphoagglutination techniques. Similar elution pattern results were obtained in isolation and purification of DABG.

Lymphocyte Agglutination

Results of lymphocyte agglutination, indicating the specificity of DATG, DABG and ND Ig are shown in Table 1. ND Ig failed to agglutinate either thymic or bursal lymphocytes. DATG
# TABLE 1

**CHICKEN LYMPHOCYTE AGGLUTINATION**

<table>
<thead>
<tr>
<th></th>
<th>Thymic lymphocytes 5200 cells/mm²</th>
<th>Bursa lymphocytes 4800 cells/mm²</th>
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<tbody>
<tr>
<td></td>
<td>undiluted</td>
<td>l:1</td>
</tr>
<tr>
<td><strong>NDIG</strong></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>DABG</strong></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>DATG</strong></td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td><strong>NDIG</strong></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>DABG</strong></td>
<td>1+</td>
<td>-</td>
</tr>
<tr>
<td><strong>DATG</strong></td>
<td>-</td>
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</tbody>
</table>
caused agglutination of thymic lymphocytes and contained no agglutinating antibodies to bursal lymphocytes. DABG caused agglutination of bursal lymphocytes, but did not contain any cross-reacting antibody to thymic lymphocytes. These results indicate that DATG and DABG were specific for lymphocytes of the thymus and bursa, respectively.

**Skin Graft Rejection**

The results of skin allograft rejection by 8 to 10 week old chickens treated *in ovo* with: (1) DATG, (2) DABG, (3) ND Ig, (4) saline are shown in Table 2. The graft rejection time of DATG was 9.4 ± 1.4 days as compared to chickens treated with saline which was 8.2 ± 0.7 days. This increase in allograft survival of 13% was significantly different (P = 0.05). In contrast, chickens treated with DABG rejected grafts at 8.3 ± 1.2 days, thus demonstrating no significant increase in graft survival (P = 0.40) when compared to chickens treated with saline. A significant difference (P = 0.10) was shown when comparing the times of skin allograft rejection between chickens treated with DABG and DATG. This finding indicated that the cell mediated response of chickens treated with DABG was normal whereas the cell mediated response of the chickens treated with DATG was impaired. The graft rejection time of 8.8 ± 1.3 days of the ND Ig treated group showed no significant differences in skin graft rejection time when compared to chickens treated with DATG (P = 0.40).
TABLE 2
SKIN GRAFT REJECTION IN CHICKENS TREATED WITH ANTITHYMUS GLOBULIN, ANTIBURSA, NORMAL DUCK IMMUNOGLOBULIN AND SALINE AT 5 DAYS OF INCUBATION

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Saline</th>
<th>DATG</th>
<th>DABG</th>
<th>ND Ig</th>
</tr>
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<tbody>
<tr>
<td>No. of animals</td>
<td>6</td>
<td>12</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Mean rejection time</td>
<td>8.2 days</td>
<td>9.4 days</td>
<td>8.3 days</td>
<td>8.8 days</td>
</tr>
<tr>
<td>S.D. of means</td>
<td>± 0.75</td>
<td>± 1.37</td>
<td>± 0.86</td>
<td>± 1.25</td>
</tr>
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</table>
Humoral Response to Brucella abortus

The humoral response of chickens treated with DABG and saline in ovo are shown in Figure 2. The chickens treated with DABG were tested at 4 weeks after stimulation and demonstrated a mean antibody titer to B. abortus of $11.2 \pm 17.2$, at 8 weeks a mean antibody titer of $12 \pm 8.3$ and at 10 weeks a mean antibody titer of $25 \pm 22$. In contrast, the chickens treated with saline had a significantly higher immune response as demonstrated by a mean antibody titer to B. abortus of $49 \pm 46$ at 4 weeks after stimulation and at 8 weeks a mean antibody titer of $185 \pm 263$ and at 10 weeks a mean antibody titer of $362 \pm 363$.

The humoral immune response by 12 week old chickens treated with DATG and saline in ovo are shown in Figure 3. The mean antibody titer to B. abortus of $1250 \pm 800$ produced by the chickens treated with the DATG was not significantly different ($P = 0.40$) from the mean titer of $1180 \pm 450$ of chickens treated with saline.

The immune response of 6 week old chickens treated with NDIg and saline in ovo is shown in Figure 5. The chickens treated with NDIg demonstrated a mean antibody titer of $97 \pm 173$, which was indistinguishable from the mean antibody titer of $97.5 \pm 149.6$ for chickens treated with saline after stimulation with B. abortus. There was no significant difference ($P = 0.40$) in mean antibody titer of chickens treated with NDIg and saline.

The chickens treated with saline, DATG and NDIg demonstrated a humoral immune response to B. abortus, while the chickens treated
with DABG were markedly deficient in their response after repeated challenges of \textit{B. abortus} antigen.

**Histological Examination**

**Thymus**: With the exception of one chicken, little or no alteration in the morphology of the thymus was observed in chickens treated with DATG, DABG or NDIg and saline. Extensive alteration of the thymus of one chicken treated with DATG was observed upon autopsy at hatching (Fig. 5). Distinct cortical and medullary regions were absent with epithelial cells making up the predominate cell type. The number of thymic lymphocytes was considerably reduced. In contrast, the normal thymus (Fig. 6) contained distinct cortical regions consisting mainly of small lymphocytes while the medullary region contained medium and large lymphocytes and epithelial cells.

**Bursa of Fabricius**: Like the thymus, little or no alteration in the morphology of the bursa was observed in chickens treated with DATG, DABG, NDIg and saline (Fig. 7). The inner structure of the bursa consisted of many longitudinal plica. The plica were lined with columnar epithelium and contained many lymphoid follicles separated by loose connective tissue. The follicles were divided into medullary and peripheral cortical zones by a basement membrane. The medullary portion of the follicle contained small, medium and large lymphocytes, while the cortical areas contained small lymphocytes.

**Spleen**: The spleen of the chicken consists of two types of lymphoid tissue, (1) the thymic-dependent lymphoid tissue seen along
the small arteries and arterioles which is composed of sheaths of small lymphocytes (Fig. 8); (2) the bursa-dependent lymphoid tissue consisting of an ovoid to round lymphoid follicle encapsulated by a fibrous sheath which lie in juxtaposition to a small artery (Fig. 9). Large and medium lymphocytes and reticular cells were found within the follicles. The spleens of chickens treated with DATG, saline and ND Ig were morphologically similar in most respects, particularly their bursal and thymic-dependent areas.

Comparison of Antibody Formation and Bursal-Dependent Areas in the Spleen: Chickens treated with DABG showed decrease in bursal dependent areas in the spleen (mean = 10.66 ± 11.36) when compared to chickens treated with saline (mean = 17.3 ± 3.97) [Student's "t" test, P = 0.15]. Comparison of antibody titer with bursal-dependent areas of chickens treated with DABG and saline revealed a high degree of correlation (P = 0.05) by the correlation coefficient test. These findings indicated a directly proportional relationship between bursal-dependent areas and antibody function. Further, it demonstrated the functional deficits associated with in ovo treatment of DABG.
DISCUSSION

The results of the present study indicate that antisera against thymic and bursal lymphocytes can be demonstrated after extensive cross-absorption on solid immunoabsorbent columns. Using anti-chicken lymphocyte globulins directed against bursal or thymic lymphocytes, it is possible to functionally alter humoral or cell mediated immunity, thus providing further evidence for the dichotomy of the immune function in chickens.

The specificity of thymic or bursal antisera is in agreement with the results reported by Forget et al. (10) who were able to demonstrate specific antisera against thymic or bursal lymphocytes after extensive cross-absorption with bursal and thymic cell suspensions. The present study differs from the findings reported by Jankovic et al. (16), who were unable to demonstrate antigenic differences between thymic and bursal lymphocytes. It is probable that their findings were due to incomplete cross-absorptions by using chicken erythrocytes and lyopholized thymus and bursa lymphocytes. It is possible that the denaturation effect of lyopholization of the bursal and thymic lymphocytes could result in loss of antigenic specificities. It has been shown by Aoki et al. (1) that the isoantigen and H-2 antigens in mice are localized on the cell membrane.
Antigens associated with cell membranes have been shown to very labile subject to denaturation (18).

These results demonstrate for the first time that the thymic-dependent immune response can be altered by administration of DATG during embryogenesis. Prior to this study, the surgical ablation of the thymus of the chicken at hatching was the earliest that manipulation of the thymus had been reported (15, 18). Surgical neonatal thymectomy causes suppression of cell-mediated immune function, i.e., homograft rejection and auto-immune diseases. Likewise, in the present study, the cell mediated immune responses were impaired in chickens treated with DATG. These findings are in agreement with those of Janković et al. (17), who reported functional alteration in the cell-mediated responses of chickens treated with anti-thymus globulin (ATG) at 4 weeks post-hatching.

In the present study, the humoral immune function of chickens stimulated with Brucella abortus (B. abortus) was unaffected by the DATG treatment in ovo, in contrast to the deficits demonstrated in the thymic-dependent immune response. B. abortus, a bacterium with an endotoxic cell wall is a thymic independent antigen (25) thought to be capable of stimulating antibody production by the bursal-dependent system without the involvement of the thymic-dependent system. In contrast to the present study, Janković et al. (17) reported that the humoral response of chickens treated with ATG was markedly reduced when stimulated with bovine gamma globulin (BAG).
It is quite possible that bovine gamma globulin may be a thymic dependent antigen similar to fowl gamma globulin as recently reported by Miller and Warner (24). The reduced humoral response to bovine gamma globulin may be the result of the ATG treatment on the thymic dependent lymphocytes. Also, it is quite possible that ATG utilized by Jankovic et al. (17) also reacted with bursal-dependent systems as a result of cross-reacting antibody which could cause the decreased antibody response.

Chickens treated with DABG in ovo demonstrated a marked deficiency in their humoral response to E. abortus stimulation. Even after repeated stimulations, the chickens did not develop a significant humoral immune response as exemplified by a low mean antibody titer. Corollary results have been demonstrated by Jankovic et al. (17) and Kincade et al. (20). Suppression of γM and γG immunoglobulin synthesis in chickens has been demonstrated by Kincade et al. (20) by the administration of anti-chicken γM globulins followed by bursectomy in ovo. In vitro suppression of hemolytic plaque formation in spleen cell preparations by anti-bursal globulin (ABG) have been reported by Potoworowski et al. (29). Decreased production of anti-bovine globulin by chickens treated with ABG at 4 weeks post-hatching has been demonstrated by Jankovic et al. (17).

Although the bursa-dependent system was depressed, the integrity of cell mediated responses of chickens treated with DABG
was normal. Similarly, a competent cellular immune mechanism in chickens treated with anti-chicken IgM globulin has been reported by Kincade et al. (20). These results, however, differ from the earlier attempts at hormonal bursectomy (13, 31) which also seemed to affect the cellular immune response.

Chickens treated in ovo with NDIg demonstrated a normal humoral immune response. The cell mediated response, however, was not different from the cell mediated response of chickens treated with DATG. At the present time, the mechanism of NDIg alteration of the thymic dependent immune response is not clear. A 15% incidence of eye malformation ranging from micro-ophthalmia to anophthalmia was demonstrated in chickens treated with NDIg. No eye malformations were demonstrated in either DATG or DABG treated chickens. NDIg consisted of heterologous antibodies of unknown specificity which was isolated by salt precipitation and ion exchange chromatography and subsequently absorbed with peripheral blood leukocytes of chickens, whereas, DABG and DATG were purified on the basis of antigen-antibody interaction which results in purified antibodies of known specificity. Brent et al. (5) administered antisera to pregnant rats and demonstrated a wide spectrum of malformations involving up to 86% of the total rat embryos. Subsequent findings of Bragonia et al. (4) showed that the malformation causing antibody was due to presence of anti-yolk sac antibody, which seems to act by interfering sterically with
normal yolk sac function. The loss of yolk sac function during a critical period of fetal organogenesis resulted in the malformations. An analogous situation may be present in the ND Ig in which there may be antibodies that act directly on the developing organ systems or possibly the antibodies act indirectly on the organ system by affecting yolk sac transport (4) or by interference in ion transport in the vascular and extra-vascular compartments of the developing chick embryo (12).

Changes in thymic morphology of a one-day post-hatched chicken treated with DATG at 5 days of incubation have been demonstrated for the first time. However, no alteration of bursa morphology was demonstrated in one-day post-hatched chickens treated with DABG in ovo. Jankovic et al. (16) administered ABG or ATG to chicken embryos from 7 days until 17 days of incubation and demonstrated degenerative changes in the follicles of bursa. However, they did not demonstrate any alteration in the thymus. Kincade et al. (20) showed alterations in the bursa of chickens due to the administration of anti-chicken IgM globulin at 13 days of incubation. They describe the lymphoid follicles of bursa as being smaller, sparsely populated with lymphoid cells and undergoing degenerative changes.

No changes in the morphology of the central lymphoid organs of 10 weeks post-hatched chickens treated with DATG, DABG and ND Ig, in ovo were demonstrated in the present study. Similar findings were reported by Jankovic et al. (17) in which they found no alteration of
thymus or bursa of chickens treated with ATG, ABG at 4 weeks post-hatching. In the present study, a significant decrease in bursal dependent areas in the spleens of chickens treated with DABG in ovo was demonstrated. The decreased humoral immune response of the chickens treated with DABG shows a strong correlation with the decrease in splenic bursal dependent areas. Jankovic et al. (17) demonstrated similar results in which there was a decrease in bursal-dependent areas of the spleen in chickens treated with ABG at 4 weeks post-hatching.

From the data that has been presented, it is not possible to conclusively identify the mechanism through which DATG or DABG affects the developing central lymphoid organs. It has been proposed by Moore and Owen (26) that an interaction of epithelial cells and hemopoietic stem cells is necessary for the complete organogenesis of the thymus and bursa. It is possible that DATG or DABG may affect this interaction leading to a functional alteration of the developing embryo. DATG and DABG combine with the lymphocyte after it has undergone its maturation process in a similar manner as has been shown for anti-lymphocyte globulin (ALG) in altering the immune function of the lymphocyte, i.e., blindfolding, self-sterilization, and antigen competition (14). Although the central lymphoid organs of the post-hatched chicken were morphologically normal after the in ovo treatment with DATG or DABG, there were functional deficits in the immune response. This is also the case when ALG is administered
to the adult animal in that the only morphological change is a lymphopenia.

The administration of DATG and DABG in ovo in the chicken may provide a model system for the study of immunological deficiency disorders. The bursa-dependent immune deficit resulting from administration of DABG in ovo is analogous, functionally, to sex-linked agammaglobulinemia (6) in the human. Likewise, thymic dysplasia (27) in humans manifests itself, functionally, in the same manner as the cell-mediated deficiency associated with chickens treated with DATG.
SUMMARY AND CONCLUSION

Antisera, of high specificity, was produced against thymic and bursal lymphocytes in the chicken. By the in ovo administration of the antibodies directed against central lymphoid tissues further evidence has been demonstrated for the function of the immune system in the chicken by the selective inhibition of immunologic function of thymus or bursa of Fabricius. A model system for immunological deficiency disorders found in humans of sex-linked agammaglobulinemia and thymic dysplasia can be developed by selective inhibition of central lymphoid organs in chickens through the use of specific anti-lymphocytic globulin.
PLATE I

Fig. 1

Graph of the isolation and purification of DATG.

Arrows indicate the addition of glycine-HCl, pH 2.8
buffer for the elution of proteins. See text for
details of peaks A, B, C, D.
Humoral immune response of chicken treated with DABG and saline in ovo, stimulated (S) with B. abortus at 4 weeks and challenged (C) at 6 and 10 weeks post-hatching.
TOO - SALINE TREATED CHICKENS • •
DABG TREATED CHICKENS — —

MEAN ANTIBODY TITER

SALINE TREATED CHICKENS — —
DABG TREATED CHICKENS — —

MEAN ANTIBODY TITER

WEEKS

(2)
PLATE III

Fig. 3 Humoral immune response of chickens treated with DATG and saline in ovo and stimulated with \textit{B. abortus} at 12 weeks post-hatching.
MEAN ANTIBODY TITER

SALINE TREATED CHICKENS
DATG TREATED CHICKENS

DAYS

(3)
PLATE IV

Fig. 4  Humoral immune response of chickens treated with ND Ig and saline in ovo and stimulated with B. abortus at 6 weeks post-hatching.
MEAN ANTIBODY TITER

SALINE TREATED CHICKENS
NDIG TREATED CHICKENS

MEAN ANTIBODY TITER

DAYS

(4)
PLATE V

Fig. 5 The thymus of a one-day-old chicken treated with DATG at 5 days of incubation. There is a lack of a distinct cortical and medullary region. The predominate cell type is epithelial.

Fig. 6 The thymus of a one-day-old chicken treated at 5 days of incubation with saline. There are distinct cortical (C) and medullary (M) areas.
PLATE VI

Fig. 7  
The spleen of a chicken treated with saline in ovo.  
A developing bursal dependent lymph nodule (ln) next  
to an artery (a). A thymic dependent area is indicated  
by an aggregation of dense lymphocytic tissue (dl)  
surrounding an artery.

Fig. 8  
The spleen of a chicken treated with saline in ovo.  
A well-developed bursal-dependent lymph nodule (ln)  
is shown next to an artery (a).

Fig. 9  
A lymphocytic follicle of the bursa of Fabricius. The  
follicle consists of a medullary portion (m) and a  
cortical portion (c) separated by a basement membrane  
(mm).
Isolation of Membrane Associated Antigens*

1. Homogenize the bursa or thymus with an Omni Mixer (Sorvall Company, New Haven, Connecticut) in three 20 second runs at its maximum speed in a sonication buffer consisting of (1) 0.05 M Tris HCl, (2) 0.0025 M KCl, (3) 0.008 M MgCl₂, (4) 0.15 M sucrose at pH 7.45 at 25°C.

2. Sonicate the cell suspension with a 10 KH fixed frequency probe until, upon microscopic examination, there are only a few intact cells left in the suspension. To minimize the denaturation effects, perform the sonication procedure in an ice bath.

3. Centrifuge the crude sonicated suspension in an RC-2B centrifuge (Sorvall Company, New Haven, Connecticut) for 30 minutes at 20,000 x g at 4°C.

4. Centrifuge the supernatant in an L3- 40 centrifuge (Beckman, Palo Alto, California) at 105,000 x g in a spinco 40 head for 2 hours at 4°C. with rapid acceleration and slow deceleration.

5. Concentrate the 105,000 x g supernatant with a pressure concentrator (Diaflow, Amicon, Lexington, Massachusetts) using a UM20E membrane (Amicon, Lexington, Massachusetts) until a final volume of 10-15 ml of supernatant is obtained.
9. Add the protein solution that has been previously dialyzed against 0.3M borate saline buffer (pH 8.2) to the activated sepharose and stir the mixture on a magnetic mixer for 24 hours at 4°C.

10. Allow the protein coupled sepharose mixture to settle and decant the supernatant.

11. Resuspend the mixture with 0.5M glycine in 0.3M borate saline buffer to eliminate any reactive groups on the sepharose bead.

12. Stir the mixture for 24 hours at 4°C.

13. Use a 30 ml syringe for the column in which the protein sepharose is placed.

14. Wash the immunoabsorbant column with 0.1M HCl until a zero O.D. is reached.

15. Equilibrate the immunoabsorbant column with 0.3M borate saline buffer (pH 8.2).
Preparation of Immunoabsorbent Column

1. Wash 30 to 60 ml sepharose 2B (Pharmacia Chemical) 3 times with distilled water at a force of 500-800 g for 5 minutes. The low g force is necessary in order to prevent breakage of the sepharose beads.

2. Wash 3 times with 0.1M NaHCO₃ in the same manner as step 1.

3. Cool to 0° in an ice bath:
   (a) Dimethyl formamide (DMF)
   (b) pH 10 buffer standard
   (c) 2 N NaOH
   (d) 30 ml packed sepharose 2B suspended in a final volume of 50 ml of 0.1M NaHCO₃ solution which has been adjusted to a pH 11 with 2 N NaOH.

4. Set pH meter to 0°C. and standardize the pH electrode with the pH 10 buffer.

5. Weigh out cyanogen bromide (CNBr) [Eastman Chemical] under a hood using 2.5 gm per 30 ml sepharose.

6. Dissolve the CNBr in DMF in a 1:1 ratio.

7. Add the dissolved CNBr-DMF mixture to the sepharose and stir on a magnetic stirrer for one hour at 4°C., keeping the pH between 10.8 and 11.2 with 2N NaOH.

8. Wash the CNBr activated sepharose with 4 liters of 0.3M sodium borate 0.85% saline buffer (pH 8.2) on a suction flask.
6. Couple the concentrated protein solution to sepharose 2B using the procedure described in the next appendix.

Purification of Antisera

1. Dialize the duck anti-chicken thymus antisera (DATS) or anti-chicken bursa antisera (DABS) against 0.3 M Na borate saline buffer (pH 8.2) for 24 hours at 4°C.

2. Place DATS on thymic immunoabsorbant column at a flow rate of 15-20 cc/hr. using a peristaltic pump (Extracorporeal and Medical Specialities Company, Medford, New Jersey).

3. Then wash column with 0.3 M Na borate saline buffer (pH 8.2) until zero O.D. is reached.

4. Elute the specific antibodies from the column using a 0.05 M glycine HCl buffer (pH 2.8) at a flow rate of 150 cc/hr.

5. Bring the pH and molarity of the eluent immediately to pH 8.2 by using 0.6 M sodium borate 1.7% saline buffer (pH 8.2).

6. Concentrate the antibody mixture to 10 ml volume with a Colloidin Bag Concentrator (Schleicher and Schuell Company, Keene, New Hampshire).

7. Remove cross-reactivity of DATG by using a bursa-coupled immunoabsorbant column as above.

8. Collect the antibodies that do not adhere to the immunoabsorbant column and concentrate to a final volume of 2-3 ml by a Colloidin Bag Concentrator.

9. The DABS's are purified in exactly the same manner, however, the bursa-coupled column is used for the initial purification.
APPENDIX D
Lymphocyte Agglutination

Lymphocyte suspension of thymus and bursa:

1. Remove surgically the thymus and bursa from the chicken.
2. Remove all fat and non-lymphoid tissue from the organs.
3. Mince the tissue with scissors.
4. Homogenize the tissues in an Omni Mixer by three 20 second speed runs.
5. Filter the cell suspension through 100 mesh/inch stainless steel screen to remove cell clumps.
6. Wash the cell suspension three times at 800 x g for 10 minutes.
7. Dilute the cells to a final concentration of \(4.5-5.5\times10^3\) cells/cc.

Serum:

1. Inactivate amount needed for test at 56°C. for 30 minutes.
2. Make necessary dilutions with saline using 0.1 ml or 0.2 ml.

Containers:

1. Put clean glass slide in clean plastic box.
2. Dip brass mold into hot paraffin, then press on slide. Hardens in just a few minutes.
3. Pour just enough mineral oil (from a beaker) into boxers to cover rings. Too much causes cells to slip around and too little causes cells and serum to dry up.
Test Procedure:

1. Add 5 microliters serum to ring, making sure you place it on the glass slide.

2. Add 5 microliters cells to serum and mix with the needle point.

3. Incubate in 37°C. hot air oven on mixer at 88 rpm for 15 minutes.

4. Examine for agglutination.

Injections of Purified Immunoglobulin into Chick Embryos

1. Dialize DABG, DATG, and NDIG against Kaplan saline (7.29 gm NaCl, 0.39 gm KCL/1000 ml) for 24 hours at 4°C.

2. Sterilize all solutions by passage through swinnex filters (Millipore Corporation, Bedford, Massachusetts) into sterile vacu-containers (BD) and keep frozen until they are to be used.

3. Keep the 5 day chick embryos under standard incubation conditions.

4. Locate the allantois of the embryo by candling the egg and marking the shell with a pencil.

5. Sterilize the site of injection on the eggshell by swabbing with an iodine solution followed by 70% ETCH.

6. Cut a straight opening 4-6 mm long and to a depth to reach the shell membrane with a flame sterilized hacksaw blade over the site of the injection.

7. Using a glass cannula, draw up 10 microliters of purified immunoglobulin solution. Inject the material into the allantois by going through the opening in the shell.

8. Seal the egg with liquid paraffin and return to the incubator.

9. 24 hours after injection, check the embryo for mortality.

10. Allow chick embryo to hatch.
**Antigenic Stimulation and Antibody Response in Chickens to Brucella Abortus Antigen**

1. Inject chickens of 4, 6, 8 and 10 weeks of age intramuscularly with 1 ml of $1 \times 10^6$ *Brucella abortus* cells.

2. On 3rd, 6th and 9th day after antigenic stimulation bleed chickens for whole blood which is allowed to clot, and stored at 4°C. for 12-16 hours.

3. Inactivate the chicken sera complement by incubation in a waterbath at 56°C. for 30 minutes.

4. Titrate each serum sample by serial dilutions with the initial tube containing 0.1 ml of serum and 0.1 ml of 0.9% saline.

5. To each serum dilution add 0.1 ml of $1 \times 10^6$ cells/cc of *B. abortus*.

6. Incubate the antibody-antigen mixture at 37°C. for 1 hour.

7. Centrifuge the tubes containing the antibody-antigen mixture in a SeroFuge (Clay Adams, Parsippany, New Jersey) for 45 seconds.

8. Read tubes for presence or absence of agglutins to determine warm agglutins to *B. abortus*.

9. Incubate the above tubes at 4°C. for 24 hours and repeat steps 7 and 8 to determine the presence of cold agglutins.
Determination of the Mean Bursal Dependent Areas per Spleen Section

1. Count the number of bursal dependent areas in 6 to 8 sections of spleen and determine their mean and S.D.

2. Standardize the area of the spleen section by the following method:
   a - make a camera lucida drawing of a spleen section of each chicken.
   b - determine the area of the spleen by either polar planimeter measurements or by weighing the spleen drawing (assuming that each sheet of paper is of equivalent weight).
   c - use one chicken spleen as an arbitrary reference and set its area to 1 by dividing the area by its area.
   d - divide the areas of the other spleen sections by the area of the reference spleen.
   e - this value is the standardized spleen area for each chicken.

3. For each chicken spleen divide the mean bursal dependent areas by the standardized spleen area to determine the mean bursal dependent areas per spleen section of each chicken.

4. Use correlation coefficient test in comparing antibody titer and mean bursal dependent areas of chicken treated with DABG and saline.

5. Use Student's "t" test to compare mean bursal dependent areas of chicken spleen of chickens treated with DABG and saline.
LITERATURE CITED


