SPECIFICITY OF ALLERGIC RESPONSES FOLLOWING INJECTION

OF SIMPLE CHEMICAL PROTEIN CONJUGATES

APPROVED:

[Signature]
Major Professor

[Signature]
Minor Professor

[Signature]
Director of the Department of Biology

[Signature]
Dean of the Graduate School
SPECIFICITY OF ALLERGIC RESPONSES FOLLOWING INJECTION
OF SIMPLE CHEMICAL PROTEIN CONJUGATES

THESIS

Presented to the Graduate Council of the
North Texas State University in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

George Edward Lowke, B. S.

Denton, Texas
June, 1965
TABLE OF CONTENTS

LIST OF TABLES ........................................ iv

Chapter

I. INTRODUCTION ........................................ 1

II. MATERIALS AND METHODS ............................... 9

   Animals
   Antigen Preparation and Immunization
   Measurement of Immune Response
   Skin Tests
   Passive Transfer of Leucocytes
   Specificity Determinations by Immune Tolerance

III. RESULTS ............................................... 19

   Antibody Titration and Cross Reactions
   Skin Tests
   Immune Tolerance Experiments

IV. DISCUSSION .......................................... 28

V. SUMMARY ............................................... 35

BIBLIOGRAPHY ........................................... 37
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Precipitin Titers of Serum From Immunized Animals</td>
<td>20</td>
</tr>
<tr>
<td>II. Cross Reactions Observed in In Vitro Precipitin Tests</td>
<td>21</td>
</tr>
<tr>
<td>III. Skin Test Results of Actively Immunized Animals</td>
<td>23</td>
</tr>
<tr>
<td>IV. Skin Test Results of Passively Immunized Recipients</td>
<td>25</td>
</tr>
<tr>
<td>V. Diameter in Millimeters of Passive Cutaneous Anaphylaxis Reactions Using Serum From Immunized Animals</td>
<td>26</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION

For a considerable period of time after the discovery of serological phenomena, a suitable method was lacking for systematic investigation of chemical specificity in serological reactions. A workable approach was found when it proved possible to prepare conjugated antigens containing specifically reacting components of known constitution by attaching simple chemical compounds (haptens) to proteins (7, p. 156). According to Landsteiner's classical tenet (7, p. 158), an animal immunized with a homologous protein conjugated to a haptenic group will respond with antibody formation against the haptenic group exclusively. The homologous "carrier" protein was assumed to be inert as far as the specificity of the immune response is concerned. Recently this view has been modified by several investigators. Benacerraf and Gell (1,2,6), using picryl chloride conjugated to bovine gamma globulin, egg albumin, human serum albumin, gelatin, guinea pig gamma globulin and guinea pig serum, reported that immediate hypersensitivity (Arthus reaction) is specific for the haptenic determinant of the conjugate.
Delayed hypersensitivity is less specific and represents the response to the protein moiety of the conjugate.

In an attempt to analyze the nature of this apparent "carrier protein" specificity, Leskowitz (8) carried out studies with conjugates made with oxidized or reduced proteins. These differ from the native proteins only in tertiary or folded structure but retain the same primary or amino acid sequence. Results obtained with these materials suggested strongly that specificity of reaction to hapten-protein conjugates was to a large extent influenced by amino acid sequence. Specificity was also characterized by considerable heterogeneity, probably related to multiple antigenic determinants produced by differences of amino acid sequence in various proteins. Because of this heterogeneity, Leskowitz (9) decided to use as a more homogeneous carrier a polymer consisting of a single amino acid, tyrosine, to which he conjugated diazotized arsanilic acid. He skin tested the immunized animals with several different conjugated proteins and found positive reactions correlating to some extent with their tyrosine content.

Benacerraf and Levine (3) state that the carrier protein contributes importantly to the specificity of delayed hypersensitivity reactions. Antibodies formed later are specific
for the hapten since they react in vitro with all protein conjugates of the same hapten. The immediate hypersensitivity reactions such as Arthus and passive cutaneous anaphylaxis, mediated by these serum antibodies, showed an immunological specificity also limited to the hapten. The severity of these reactions depended on the number of hapten groups on the challenging antigen. They concluded that insofar as immediate reactions are concerned, the antibodies responsible for these reactions show a complete lack of specificity for the protein and that delayed hypersensitivity is a qualitatively different process from that involving immediate reactions.

In contrast, Salvin and Smith (11,12), Salvin (13), and Coe and Salvin (4), feel that delayed hypersensitivity has a more primitive type of specificity (specific for broad areas of the protein molecule), than Arthus reactions and circulating antibody, and that delayed hypersensitivity is merely an immature step in the formation of circulating antibody. They showed that sensitizing guinea pigs with picryl chloride and 1-fluoro-2,4-dinitrobenzene conjugated to avian egg albumins, solubilized guinea pig skin and bovine gamma globulin resulted in delayed hypersensitivity to the conjugate, to the homologous protein and to the homologous protein with a heterologous
hapten. Circulating antibody and Arthus reactions occurred subsequently specific for the conjugate as well as for the same hapten attached to a different protein. Thus delayed hypersensitivity seemed associated with the protein moiety and Arthus responses with the hapten. In animals sensitized with hen egg albumin, they noted anamnestic responses against both portions of the antigen. From this they deduced that delayed hypersensitivity is a preliminary and immature step in the immune process.

Trakatellis, Stinebring and Axelrod (14), using picryl chloride conjugated to bovine gamma globulin and human gamma globulin, both native and denatured, present data which indicate that picrylation of gamma globulin suppresses both its ability to induce anaphylactic reactivity and delayed hypersensitivity to the carrier protein. In these experiments they immunized guinea pigs with unconjugated gamma globulin and challenged them with picrylated proteins. They feel that the inhibitory effects of picrylation on delayed and immediate hypersensitivity indicates that picrylation blocks antigenic sites responsible for both types of sensitivity and that these findings cannot be reconciled with the belief that these two phenomena can be dissociated with respect to their determinant sites as indicated by Benacerraf and Gell
(1,2,6) and Benacerraf and Levine (3). Agreeing with Trakatellis, et al. (14) that the immediate and delayed types of hypersensitivity to a simple chemical protein conjugate are related immunologically are Feingold, Benjamin and Shimizu (5). Utilizing yellow jacket venom sacs and the enzyme acetylcholinesterase conjugated with picryl chloride, they offer evidence which indicates that delayed hypersensitivity is the manifestation of a weak or early response to antigenic determinants consisting of the hapten plus parts of the protein molecule. If the stimulus of this determinant is increased, the response to it is increased and manifests itself either by immediate plus delayed reactivity or immediate reactivity only. Nachtigal and Feldman (10) also present evidence that specificity of precipitating antibodies depends on both the haptenic group and the carrier protein molecule. By irradiation with X-ray followed by injection of unconjugated human serum albumin, rabbits were made unresponsive to this protein. These animals were then immunized with sulphanil-azo conjugates of human serum albumin. At the same time, these workers immunized a non-irradiated group of rabbits with conjugated rabbit serum. Animals from each group reacted to the sulphanil-azo determinant, forming two types of antibodies. One type
cross-reacted with sulphamyl-azo proteins of the homologous and heterologous carriers and one was specific to the conjugate of the homologous protein. Their conclusion was that the formation of antibodies specific for the homologous conjugates indicates that hapten conjugation resulted in the formation of an antigenic determinant comprising both the haptenic group and part of the protein carrier.

The purpose of this investigation has been to determine the characteristics of the immune response to 1-fluoro-2,4-dinitrobenzene when this hapten is conjugated with various types of proteins. In the previous reports, the use of different chemicals, protein carriers, routes of injection, antigen doses and animals has resulted in two opposing views; one states unequivocably that the specificity of immediate hypersensitivity is against the hapten and that the protein carriers play absolutely no part in the reaction, while the other suggests that the antibodies mediating the immediate type phenomena are specific for the hapten plus parts of the protein molecule.
CHAPTER BIBLIOGRAPHY


CHAPTER II

METHODS AND MATERIALS

Animals

Albino rabbits weighing two-three kilograms were used for antibody production. For the passive cutaneous anaphylaxis and immunological tolerance experiments, guinea pigs of the Hartley strain obtained from Pine Ridge Caviary, Clinton, Tennessee, and weighing 300-500 grams were used. All animals were maintained in separate cages on Purina rabbit and Purina guinea pig chow respectively, and allowed to have food and water ad libitum.

Antigen Preparation and Immunization

The proteins used in these experiments were: (a) autologous rabbit serum, (b) homologous rabbit serum, (c) heterologous guinea pig serum (pooled from several animals), and (d) heterologous hen egg albumin (salt free, twice crystallized ovalbumin obtained from Nutritional Biochemicals, Cleveland, Ohio). The hapten used was alcohol recrystallized 1-fluoro-2,4-dinitrobenzene obtained from Matheson Coleman and Bell Chemical Company, Norwood, Ohio.
Before conjugation, the protein concentration of the animal serum was measured by the biuret method as given by Gornall, Bardawill, and David (2). After measurement, the serum proteins were diluted in 0.15 molar sodium chloride to a volume of fifty milliliters of solution containing five milligrams per milliliter. Since the ovalbumin was in crystalline form, 250 milligrams were weighed on an analytical balance and dissolved in fifty milliliters of 0.15 molar sodium chloride. To each protein solution was added 0.2 milliliters of the alcohol recrystallized 1-fluoro-2,4-dinitrobenzene. The pH was then adjusted to pH 8.0 using sodium bicarbonate. The alkaline solutions were incubated at room temperature for two hours followed by overnight at four degrees C. At the end of this incubation period, each solution was placed in cellophane tubing and dialyzed against 0.15 molar sodium chloride in the cold using a magnetic stirrer. The dialysis bath was changed every two-six hours, and was changed twelve-sixteen times to remove all unconjugated 1-fluoro-2,4-dinitrobenzene. Solutions were brought back to original volume by evaporation and placed in chemically clean serum bottles.

For injection of these conjugates, hair was clipped from one side of the rabbits, and all injections were made intradermally in the same local area. An antigen dose of
0.1 milliliter of each conjugate was injected daily for six days followed by one day of rest. This schedule was repeated until thirty injections had been given totaling fifteen milligrams of protein. Each antigen was injected in duplicate so that a total of eight rabbits were used. Blood samples were taken at scheduled intervals by venipuncture for antibody determination.

Measurement of Immune Response

Circulating antibodies were measured by the ring precipitin and agar gel diffusion precipitation tests. The ring test consisted of layering diluted antigen over undiluted antiserum in capillary tubes so that an interface was obtained. The antigens were diluted in two-fold steps to a dilution of $1.52.4 \times 10^6$. If a precipitating antibody was present, a ring of precipitation would appear at the interface within two hours. In the agar-gel diffusion technique petri dishes containing "Ion agar" number two at one per cent concentration with added phenol to prevent bacterial growth, were used. Seven wells were cut in the agar plates so that a pattern was obtained consisting of a center well surrounded by six outer wells. To the center well was added undiluted antiserum and to the outer wells was added the two-fold dilutions of
antigen. Before the petri dish covers were replaced, a moistened filter paper was placed over the top to prevent drying. The plates were then incubated upright at room temperature for four days. If a precipitating antibody was present, a line of precipitate would be seen between the center and outer wells. To check for cross reactions, each antiserum was set up against each protein, both conjugated and unconjugated.

Since passive cutaneous anaphylaxis is a more sensitive means of measuring circulating antibody than any precipitin test, and since several investigators said they found cross reactions, it was decided to test all antisera against all the antigens by this method. The procedure used was that given by Campbell, Garvey, Cremer and Sussdorf (1, pp. 216-217), with slight modification. A total of sixteen guinea pigs were used (two for each antigen). The antisera used were those produced in the rabbits, and all four proteins, both conjugated and unconjugated were used as antigens. The antigen solutions were made up in 0.15 molar sodium chloride with a concentration of 0.2 milligrams of antigen per milliliter and ten milligrams Evans blue per milliliter. The backs of the guinea pigs were freed from hair by electric clippers and the cleared skin areas were wiped with seventy
per cent ethyl alcohol and allowed to dry. One-tenth milliliter of each of the eight antisera were then injected into separate locations using twenty-seven gauge needles. A saline control in the same amount was injected at the same time. Four hours later one milliliter of each antigen solution was injected intracardially. The antiserum injection sites were observed continuously for thirty minutes. A positive reaction consisted of marked bluing of the injection area, indicating an antigen-antibody reaction, and the diameter of each was measured in millimeters.

**Skin Tests**

Within five days of the end of the injection schedule, the rabbits were skin tested by injection of (a) protein carrier alone; (b) specific 1-fluoro-2,4-dinitrobenzene protein conjugate; and (c) by painting one drop of 0.5 and 0.75 per cent 1-fluoro-2,4-dinitrobenzene in olive oil on the skin. In some experiments, animals were cross tested by injection of non-specific 1-fluoro-2,4-dinitrobenzene conjugates. To accomplish these skin tests, hair was clipped from the side opposite the immunization injections. The challenging injections, in the amount of 0.1 milliliter, were given intradermally at a distance of two inches from each
other. The unconjugated hapten was painted on the skin on the same side at a distance of approximately three inches from each other and from the injection sites.

The immediate reaction (Arthus) was indicated by early inflammation and edema progressing to necrosis after twenty-four hours. Delayed reaction was read as erythema and induration at twenty-four hours. The grading systems for these reactions was as follows. Delayed: ++++, marked homogenous erythema; ++, homogeneous erythema, +, patchy erythema; +, slight erythema, and 0, no reaction. Arthus: ++++, marked hemorrhagic necrosis; ++, severe edema with necrosis and hemorrhage; +, severe edema and slight hemorrhage; +, definite edema; and +, slight edema.

Passive Transfer of Leucocytes

To confirm the delayed reactions observed in the immunized animals, and to establish that the delayed reaction was present in cases where it was masked by the Arthus reaction, peritoneal exudative cells were transferred to normal recipient rabbits. To evoke the peritoneal exudate, fifty milliliters of sterile mineral oil (Fisher Scientific Co., Paraffin Oil viscosity 125/135) was injected into the peritoneal cavity of immunized animals at the time skin tests were read. Seventy-two hours after oil injection, animals were exsanguinated by
intracardial puncture. The abdominal wall was opened and washed four times with Hanks balanced salt solution (3) modified by the addition of 0.1 per cent gelatin.

Cells thus collected were sedimented by centrifugation at 2,000 revolutions per minute for twenty minutes. Packed cells were washed once in Hanks solution and the volume recorded. Cells were resuspended in ten milliliters of Hanks solution for injection into the peritoneal cavity of the recipient rabbits. Forty-eight hours after injection, the recipients were skin tested in the manner described above. Skin tests were read twenty-four hours later.

Specificity Determinations by Immune Tolerance

Since Weisberger, Daniel and Hoffman (4), had demonstrated that large doses of chloramphenicol would suppress the primary immune response, but would have no effect on the secondary or anamnestic response, it was felt that their techniques could be integrated with the immune systems of the present experiments to strengthen the results obtained.

Chloramphenicol (Parke, Davis and Co., Detroit, Michigan) was prepared as a suspension with carboxymethyl cellulose in 0.75 per cent sodium chloride in the proportion of one gram of chloramphenicol and ten milligrams of carboxymethyl
cellulose in three milliliters of the saline. This suspension was injected intramuscularly into four guinea pigs daily for ten days. Two of the guinea pigs received 0.3 milligrams of chloramphenicol per kilogram of body weight per day, and two pigs received 0.5 milligrams per kilogram per day. The initial antigen solutions used were 1-fluoro-2,4-dinitrobenzene conjugated to egg albumin and guinea pig serum. These were made up in a concentration of four milligrams per milliliter and emulsified with an equal volume of Freund's incomplete adjuvant (Difco). Twenty-four hours after initiation of the chloramphenicol injections, one guinea pig receiving 0.3 milligrams per kilogram per day of chloramphenicol and one receiving 0.5 milligrams per kilogram per day were injected with four milligrams of the antigen solutions, two milligrams in each hind foot pad.

After completion of the chloramphenicol injections, the guinea pigs were bled weekly for several weeks to determine if antibodies had been produced against the conjugates. Twelve weeks after the completion of the chloramphenicol injections, the animals were given another injection of antigen in the same manner as before with two exceptions. Instead of the conjugated protein, they were injected with the specific unconjugated protein subcutaneously. The animals were then
bled every third day for two weeks to check for antibodies against either or both the conjugated and unconjugated proteins.
CHAPTER BIBLIOGRAPHY


CHAPTER III

RESULTS

Antibody Titration and Cross Reactions

The antibody titers from four groups of rabbits are shown in Table I. These are final titers obtained against the specific antigen used for immunization. In general, the more foreign the protein used for immunization, the higher the precipitin titer. All values are expressed as the highest dilution at which precipitation occurred.

In Table II are shown results of cross testing the antisera produced against all four proteins used for antigen production. Conjugated and unconjugated proteins were tested by both titration techniques. For clarity, positive reactions are indicated by + instead of dilution titer. Cross reaction titer was considerably lower than the titer against the specific antigen in every case. For example, the antiserum produced using conjugated egg albumin as an antigen reacted with its specific antigen to a titer of 1,52,441,600. This antiserum cross reacted with other conjugated antigens, but the titers obtained were no higher than 1,800.
### TABLE I

**PRECIPITIN TITERS OF SERUM FROM IMMUNIZED ANIMALS**

<table>
<thead>
<tr>
<th>Animals</th>
<th>Ring Test</th>
<th>Agar Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>1:51,200</td>
<td>1:16</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>1:409,600</td>
<td>1:32</td>
</tr>
<tr>
<td>Group II&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>1:819,200</td>
<td>1:32</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>1:1,638,400</td>
<td>1:32</td>
</tr>
<tr>
<td>Group III&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>1:1,638,400</td>
<td>1:512</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>1:1,638,400</td>
<td>1:512</td>
</tr>
<tr>
<td>Group IV&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>1:1,638,400</td>
<td>1:128</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>1:52,441,600</td>
<td>1:256</td>
</tr>
</tbody>
</table>

<sup>a</sup>Immunized with Autologous rabbit serum-1-fluoro-2,4-dinitrobenzene conjugate.

<sup>b</sup>Immunized with Homologous rabbit serum-1-fluoro-2,4-dinitrobenzene conjugate.

<sup>c</sup>Immunized with guinea pig serum-1-fluoro-2,4-dinitrobenzene conjugate.

<sup>d</sup>Immunized with egg albumin-1-fluoro-2,4-dinitrobenzene conjugate.
TABLE II
CROSS REACTIONS OBSERVED IN IN VITRO PRECIPITIN TESTS

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Auto. Serum</th>
<th>Auto. Serum</th>
<th>Homo. Serum</th>
<th>Homo. Serum</th>
<th>G.P. Serum</th>
<th>G.P. Serum</th>
<th>E.A. Serum</th>
<th>E.A. Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Auto. DNFB</td>
<td>Homo. DNFB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*a Immunized with autologous serum conjugate.  
*b Immunized with homologous serum conjugate.  
*c Immunized with egg albumin conjugate.  
*d Immunized with guinea pig conjugate.
It should be noted in Table II that each antiserum, in addition to precipitating its specific conjugated antigen, also precipitated the specific unconjugated protein from which its conjugated antigen was made, with one exception. The unconjugated autologous serum was not precipitated. It should also be noted that only the antibody against the egg albumin conjugate precipitated the other three conjugates, but not the unconjugated proteins. Cross reactions with either conjugates or untreated proteins were not seen with other antisera.

Skin Tests

Results of skin testing the immunized animals are given in Table III. The first two columns show results of painting two different concentrations of 1-fluoro-2,4-dinitrobenzene on the skin. The second two columns are results of the intradermal injections of the unconjugated protein carriers and the antigens used for injections. There was no Arthus or delayed hypersensitivity observed with the unconjugated autologous or homologous proteins whereas strong reactions were seen with the two heterologous proteins. Contact sensitivity specific for the chemical hapten was seen in all animals. In cases where both Arthus and delayed reactions
### TABLE III

**SKIN TEST RESULTS OF ACTIVELY IMMUNIZED ANIMALS**

<table>
<thead>
<tr>
<th>Immunized Animals</th>
<th>1-fluoro-2,4-dinitrobenzene Concentration</th>
<th>Unconj. Protein</th>
<th>Conj. Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5%</td>
<td>0.75%</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group I&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit 1</td>
<td>++</td>
<td>++</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>+++</td>
<td>+++</td>
<td>0</td>
<td>+++</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group II&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit 1</td>
<td>+</td>
<td>+++</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>+++</td>
<td>+++</td>
<td>0</td>
<td>+++</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group III&lt;sup&gt;c&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit 1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group IV&lt;sup&gt;d&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit 1</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

<sup>a</sup>Immunized with Autologous rabbit serum-l-fluoro-2,4-dinitrobenzene conjugate.

<sup>b</sup>Immunized with Homologous rabbit serum-l-fluoro-2,4-dinitrobenzene conjugate.

<sup>c</sup>Immunized with guinea pig serum-l-fluoro-2,4-dinitrobenzene conjugate.

<sup>d</sup>Immunized with egg albumin-l-fluoro-2,4-dinitrobenzene conjugate.
were present, Groups III and IV, separate delayed reaction could not be distinguished.

To establish that the delayed reaction was present in these cases, peritoneal exudate cells were transferred to normal rabbits. The results of skin tests on recipient animals are given in Table IV. All reactions shown are delayed reactions since immediate hypersensitivity is not transferred by leucocytes. Again, no delayed reaction is observed specific for autologous or homologous serum proteins. The delayed reaction was observed in all tests involving the chemical hapten and when the protein was foreign.

Passive Cutaneous Anaphylaxis

In passive cutaneous anaphylaxis experiments, all antisera produced were tested against all the proteins used as antigens, both conjugated and unconjugated. The results shown in Table V are measurements in millimeters indicating the diameter of the area of marked bluing resulting from antigen-antibody reaction.

It should be noted that results correlate quite well with the precipitin cross reactions. There are however, more cross reactions shown by this technique than by the precipitin technique as would be expected from this more sensitive procedure.
## TABLE IV

**SKIN TEST RESULTS OF PASSIVELY IMMUNIZED RECIPIENTS**

<table>
<thead>
<tr>
<th>Recipient Animals</th>
<th>Volume Peritoneal Exudate</th>
<th>1-fluoro-2,4-dinitrobenzene Concentration 0.5%</th>
<th>Unconj. Protein</th>
<th>Conj. Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I(^a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>1.3 cc</td>
<td>++</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>0.3 cc</td>
<td>+</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>Group II(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>0.4 cc</td>
<td>++</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>Group III(^c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>0.3 cc</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Group IV(^d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>1.0 cc</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>0.5 cc</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\) Recipients of Group I (Autologous conjugate)

\(^b\) Recipients of Group II (Homologous conjugate)

\(^c\) Recipients of Group III (guinea pig conjugate)

\(^d\) Recipients of Group IV (egg albumin conjugate)
### TABLE V

**DIAMETER IN MILLIMETERS OF PASSIVE CUTANEOUS ANAPHYLAXIS REACTION USING SERUM FROM IMMUNIZED ANIMALS**

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Group I Rabbit 1</th>
<th>Group II Rabbits 1 &amp; 2</th>
<th>Group III Rabbits 1 &amp; 2</th>
<th>Group IV Rabbits 1 &amp; 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Group IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit 1</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Rabbit 2</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>
Immune Tolerance Experiments

By the technique described in the preceding chapter, the immune response against the two conjugated proteins was suppressed completely by both dosages of chloramphenicol. The guinea pig receiving 0.5 milligram per kilogram chloramphenicol per day made unresponsive to egg albumin 1-fluoro-2, 4-dinitrobenzene conjugate expired before receiving the second injection. The other guinea pig made unresponsive to conjugated egg albumin, upon injection of unconjugated egg albumin, died from anaphylactic shock. This was evidenced by hemorrhage in his liver and by extreme lung congestion seen at autopsy. The two pigs made unresponsive to conjugated guinea pig serum then injected with unconjugated guinea pig serum responded by production of antibodies which precipitated both the conjugated and unconjugated antigens.
CHAPTER IV

DISCUSSION

It has been shown in these experiments that high titer precipitins can be produced in rabbits by injecting antigens composed of a simple chemical, 1-fluoro-2,4-dinitrobenzene conjugated to various proteins. Precipitins were produced in high titer even when the antigen was composed of 1-fluoro-2, 4-dinitrobenzene conjugated to a rabbit's own normal serum. Results obtained by cross testing the four groups of antisera against the four conjugated and unconjugated proteins do not agree with results reported by other investigators (1,2,3,4, 6,10,11,12). These investigators feel that immediate hypersensitivity, mediated by precipitating antibodies, is specific for the hapten, and that the protein carriers play no part in the reaction. Results shown in Tables II and V indicate that the protein carriers do contribute to the specificity of the precipitin reaction. All the antisera precipitated with specific unconjugated proteins except in the case of the autologous rabbits. Since the unconjugated autologous serum is normal serum, drawn from the rabbit before immunization
was started, immune response specific for proteins in this serum would not be expected unless the protein was considerably altered on removal from the animal body, which is not the case. The antiserum obtained from the animals immunized with conjugated egg albumin precipitated all conjugates, indicating one of two possibilities. Either the antibodies produced in response to conjugated egg albumin are specific for the hapten alone or egg albumin has within its molecular structure amino acid sequences similar to sequences found in the serum albumins of other animal species. These sequences in the unconjugated albumins are not similar enough to cause cross precipitation. Addition of 1-fluoro-2,4-dinitrobenzene would add a common determinant group on all conjugates, without extensive alteration of arrangements of sequences in the protein molecules. Since cross reaction titers were considerably lower than the titers against the specific antigen, the lack of alteration of the protein appears to be borne out. This is also indicated by the size of reaction sites in passive cutaneous anaphylaxis experiments.

In the passive cutaneous anaphylaxis experiments, Table V, unconjugated autologous serum reacted weakly with conjugated autologous antisera. This strengthens the belief that in systems employed in these experiments, immediate
hypersensitivity reactions, of which passive cutaneous anaphylaxis is an example, are specific for the hapten, but are influenced by the adjacent portion of the protein molecule. Reaction of autologous serum with anti-conjugated autologous serum did not appear in the ring and agar diffusion precipitin techniques, which are much less sensitive than the passive cutaneous anaphylaxis reaction both in detection of amount of antibody and in detection of factors involved in specificity.

Other cross reactions seen in the passive cutaneous anaphylaxis experiments and not in the precipitin reactions were that the antisera against the guinea pig conjugate gave weak reactions with other conjugates but not with unconjugated serum. Such reactions indicate that the hapten, when conjugated with serum proteins, adds determinant groups that are similar enough in many animal sera to react with antibodies specific for other serum protein conjugates, but in no case are the reactions as strong as against the specific antigen. The evidence alludes to the role the protein carrier plays in specificity of the reaction.

Another major point of difference in the results obtained from these experiments and the results of other investigators, notably Salvin, Smith, and Coe (4,10,11,12) is that the results presented show that delayed hypersensitivity is
produced specific for the hapten as well as for the unconjugated protein. The above investigators state that contact allergy against the hapten is not produced in guinea pigs if the protein moiety of the conjugate is heterologous to the guinea pig. Results in Tables III and IV demonstrate that in systems used for these experiments, contact allergy against the hapten was most severe when a heterologous conjugate had been utilized. It remains to be seen whether these differences are peculiarities of the two animal species or whether they are due to differences in dosage and routes of injection.

Salvin and Smith (10,11) injected no more than 100 micrograms of antigen which was emulsified in Freund's complete adjuvant. Injections were made into the foot pads, whereas experiments presented here employed a total of fifteen milligrams of antigen injected intradermally and no adjuvant was used.

A point that is not explained by this work is the fact that although in vitro, high titer precipitins were produced in all animals, Arthus reactions were elicited only when the protein was sufficiently foreign. The failure of the anti-autologous and homologous sera to produce this reaction is inexplicable. According to Benacerraf and Gell (1,2,6) and Salvin, Smith, and Coe (4,10,11,12), these antisera should produce Arthus if as they say, the antibody is specific for
the hapten alone. This phenomenon is probably due to an apparent important role of the protein carrier molecule in the production of the Arthus reaction. Such an effect has not been reported previously.

Animals in which the primary immune response to an antigen has been suppressed by chloramphenicol will respond anamnestically to a second injection of the antigen (14). An animal made tolerant to a conjugated protein should, if the specificity of precipitating antibodies is against part of the protein and the hapten, respond anamnestically to the specific unconjugated protein given as the second injection. This was found to be the case in the present experiments and strengthens the hypothesis that precipitins are specific for the hapten plus parts of the protein molecule.

The experiments discussed tend to support the hypothesis of Feingold et al. (5), Machtigal and Feldman (9), and Leskowitz (7,8), that immune response to a simple chemical-protein conjugate involves both delayed and immediate types of hypersensitivity and that specificities of these reactions involve the hapten and are influenced by adjacent portions of the protein molecule.
CHAPTER BIBLIOGRAPHY


CHAPTER V

SUMMARY

Rabbits injected with antigens composed of 1-fluoro-2,4-dinitrobenzene conjugated to autologous, homologous and heterologous proteins responded by producing high titer precipitins (immediate hypersensitivity) and strong delayed hypersensitivity. Delayed hypersensitivity was specific for the simple chemical hapten in all cases, but was specific for the protein as well only when the protein moiety was sufficiently foreign to the rabbit.

Specificity of immediate hypersensitivity reactions was shown by cross precipitin tests and by passive cutaneous anaphylaxis and immune tolerance experiments in guinea pigs. Results of these tests indicate that the antibodies mediating immediate hypersensitivity reactions are specific for the hapten of a simple chemical protein conjugate, but are influenced strongly by the adjacent portions of the protein molecule.

These results are in direct opposition to results reported by previous investigators. However, since the animals, routes of injection and dosages used in the present
investigation are different, it remains to be seen whether
the differences in results are due to peculiarities of the
animal species or due to other factors.
BIBLIOGRAPHY

Books


Articles


