THE EFFECT OF REPEATED ANTIGEN INJECTIONS ON THE C' AND C'4 TITERS IN GUINEA PIG SERUM

APPROVED:

Major Professor

Minor Professor

Director of the Department of Biology

Dean of the Graduate School

THE EFFECT OF REPEATED ANTIGEN INJECTIONS ON THE C' AND C'4 TITERS IN GUINEA PIG SERUM

THESIS

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

For the Degree of

MASTER OF ARTS

By

Perry Owen Teague, B. A.

Denton, Texas

June, 1961

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

INTRODUCTION

The consequences of the immune response in animals after contact with an antigen have been given considerable attention since Bordet (3) in 1896 first demonstrated that a humoral factor other than specific antibodies was necessary for bacteriolysis in immune serum. He noticed that there was a thermolabile factor in this serum which influenced its ability to lyse bacteria. It was found that this factor could be inactivated by heating to 56 degrees C and thus confirmed the earlier work of Traube. Traube had observed that all fresh blood was naturally bactericidal and was influenced by the thermolability of some component of the serum. Bordet also found in his investigations that lysis by immune serum was not restricted to bacteria, but that guinea pig serum from animals injected with rabbit blood would lyse fresh rabbit red blood cells.

These early investigations established the existence of alexin, or complement, and stimulated other investigators to attempt to determine the true nature of this substance.

Zinsser (17), in 1939, reviewed the early investigations of Nuttall, Buchner, and Pfeiffer done in the years 1886-1894. Nuttall's work revealed that the lytic action of immune serum was weakened after aging. Buchner, in 1889, also demonstrated the heat lability of some portion of the blood serum. He named this substance alexin. In 1894, Pfeiffer demonstrated the bacteriolytic ability of guinea pig peritoneal fluid to lyse cholera organisms after the animals had recovered from an experimental infection of cholera. Morgenroth and Ehrlich conducted later investigations and found that the activity of complement could only be determined as the result of formation of an antigen-antibody complex, and proposed the name complement.

Ferrata, in 1907, was the first to recognize that complement does not exist as a single entity. He determined that there are at least two fractions. Today it is generally accepted that complement exists in at least four fractions designated as C'1, C'2, C'3, and C'4. Recent investigations of Brumfield (4) suggest the existence of two additional components, C'5 and C'6.

The chemical identity of the components of complement has partially been established. C'1 is a euglobulin and

is heat labile, being destroyed by heating to 50 degrees C for 30 minutes and precipitates when serum is dialyzed against water. C'2 has been identified as a muco-euglobulin and is also heat labile. Closely associated with the C'1 fraction is C'3, a euglobulin which can be differentiated from C'1 in that it is more heat stable, withstanding 56 degrees C for 20 minutes. C'3 is inactivated with yeast cells or zymosan, a carbohydrate derivative of yeast cells. The C'4 component is a muco-euglobulin closely associated with the C'2 fraction; however, it is more heat stable than C'2, tolerating 56 degrees C for 20 minutes. It is easily inactivated by ammonia or hydrazene. C'5 and C'6 have not been completely identified.

Numerous attempts have been made to discern the site of biosynthesis of complement. However, the identification of an isolated system as a source of complement has been as evasive as attempts to determine the location of antibody synthesis. At present, the liver, the leukocytes, and the reticuloendothelial system do not appear to be directly concerned with complement synthesis.

Complement has been shown to be closely associated with serum proteins. Cushing (5) states that it must be

considered as a function of serum proteins. Ecker (8) has substantiated this statement in reporting that as much as 90 per cent of the activity can be separated in a fraction of the serum globulins.

Becker (2) has recently confirmed the earlier works of Pillemer (14) as to the order of reactivity of the various components in immune hemolysis. Both have shown that C'4 is the first component fixed to the immune aggregate. This is followed by fixation of C'1 and C'2 and is a prerequisite for the binding of these two. C'3 was found to remain in solution. Becker has also demonstrated that C'1 does nothing more to the sensitized cell than to attach itself. However, he has postulated that it has an enzymatic action on C'4 and C'2 which leads to their being bound.

The importance of complement in normal and immune animals has been given much consideration, with various viewpoints being established. The necessity for complement in resistance to disease was noticed by Moore in 1919 (11). He found that a group of complement deficient guinea pigs were much more susceptible to cholera infection than were normal animals. Later, Ecker (7) found that complement is frequently reduced during the course

of an infectious disease, especially in the C'4 component. His data showed that most patients succumbed when their C'4 titer was low. This seems to indicate the importance of C'4 in the immune response. Nungester (13) has reported that there was a 34 per cent mortality in patients who had experienced a decrease in their C'4 titer. Recently Schlagenhauf (16) has shown the protective effect of C'4 by extending the average life span of lymphoid tumor infected mice beyond that of control animals by injecting them with guinea pig C'4.

Contrasting evidence has been reported as to whether or not antigen contact results in an increase in complement activity. Axelrod (1) and Dozois (6) have both demonstrated that complement activity is not stimulated by antigen injection. Axelrod, however, found that non-antigenic substances such as Panothenic acid, thiamin, biotin, riboflavin, and pyrodoxine stimulated an increase in complement. Rice (15) has also shown that dextran, a relatively poor antigen, caused no significant change in complement activity when injected into guinea pigs. In opposition to the data of Axelrod and Dozois is that compiled by Pillemer (14) and Muschel and Treffers (12).

Pillemer found that there was a slight rise in total complement after antigen injection into guinea pigs. He has stated that there is a definite non-specific role which complement plays in immunity. This statement is in agreement with the conclusion of Dozois (6) that the bactericidal power of a given serum is not only governed by the amount of antibody present, but also upon the amount of complement. Muschel and Treffers (12) also noticed rises in total complement after immunization. Guthrie (9), on the contrary, could not demonstrate a significant statistical increase in total complement after antigen injection. However, he did show considerable increases in the C'4 component after injection of Salmonella typhosa, Brucella abortus, or egg white antigen. In earlier investigations (10) he observed changes in the C'4 titer of guinea pigs after immunization which led him to conclude that the increase was due to the ability of the C'4 fraction to become more resistant to inactivation or because the immune process had caused an increase in total volume of C'4. This work appears to agree with the findings of Nungester and Ecker who have pointed out the apparent importance of C'4 in patients suffering from infectious diseases.

These reports indicating that C'4 must play a significant part in the immune response have suggested that further investigations be conducted. In this study the effects of repeated antigen injections on total complement (C') and C'4 of guinea pig serum were investigated to determine if constant antigenic stimulation would show changes in the C' and C'4 titers. Attempts were also made to correlate any changes with variations in antibody titers during the repeated antigen injections.

CHAPTER BIBLIOGRAPHY

1. Axelrod, A. E., and Pruzansky, J., "Role of Vitamins in Antibody Production," Vitamins and Hormones, XIII (1955), 14-15.

2. Becker, E. L., "Concerning the Mechanism of Complement Action. V. The Early Steps in Immune Hemolysis," Journal of Immunology, LXXXIII (September, 1960), 299-308.

3. Bordet, J., Resume of Immunity, Studies in Immunity, collected and translated by F. P. Gay, New York, John Wiley and Sons, Inc., 1901.

4. Brumfield, H. P., "A Factor of Complement Inactivation by Histamine or Ethylenediamine," Journal of Immunology, LXXII (May, 1954), 393-397.

5. Cushing, J. E., Campbell, D. H., Principles of Immunology, New York, McGraw-Hill Co., 1957.

6. Dozois, T. F., "The Role of Human Complement in Bactericidal Phenomena," Journal of Immunology, XLVII (March, 1945), 229.

7. Ecker, E. E., "Complement," Annual Review of Microbiology, II (1948), 121.

8. Ecker, E. E., Pillemer, L., Jones, C. B., and Seifter, S., "Separation of Complement from Fresh Guinea Pig Serum," Journal of Biological Chemistry, CXXXV (June, 1940), 347.

9. Guthrie, R. K., Hilton, L. H., Renshaw, L., and Whalen, P. L., "Effect of Antigen Injection on the Fourth Component of Complement," The Texas Journal of Science, XII (May, 1960), 55-60.

10. Guthrie, R. K., "A Study of the Relations Between the Blood Antiprotease System and Complement Activity in Specific and Non-Specific Immunological Reactions," unpublished doctoral dissertation, Department of Microbiology, Baylor University, Waco, Texas, 1954.

11. Moore, H. D., "Complementary and Opsonic Functions in Their Relation to Immunity," Journal of Immunology, IV (1919), 425-441.

12. Muschel, L. H., and Treffers, H. P., "Quantitative Studies on the Bactericidal Actions of Serum and Complement," Journal of Immunology, CIII (January, 1956), 20-27.

13. Nungester, W. J., "Non-Specific Factors in Immunity," Annual Review of Microbiology, VII (1954), 363-392.

14. Pillemer, L., and Ecker, E. E., "Anticomplementary Factor in Fresh Yeast," Journal of Biological Chemistry, CXXXVII (January, 1941), 139.

15. Rice, C. E., Boulanger, P., and Annau, E., "The Effect of Repeated Injections of Dextran or Polyvinylpyrolidone on Serum Complement in the Guinea Pig," Canadian Journal of Public Health, XLV (1954), 264-267.

16. Schlagenhauf, G. K., "A Study of the Role of Complement in the Protection of AKr Mice Against Transplanted Lymphoid Leukemia," unpublished master's thesis, Department of Biology, North Texas State College, Denton, Texas, 1957.

17. Zinsser, H., Enders, J. P., and Fathergill, L. D., Immunity Principles and Applications in Medicine and Public Health, 5th ed., New York, Macmillan Co., 1939.

CHAPTER II

MATERIALS AND METHODS

Immunization of Animals

The antigens to be tested were injected into healthy guinea pigs weighing from 125 to 800 grams. The animals were isolated into sex groups to avoid pregnancy since this has been shown to induce variation in the complement titer (1). Stock guinea pigs as well as test animals were kept in completely air conditioned quarters and were fed a diet composed of Purina guinea pig chow containing Vitamin C, oats, and alfalfa hay. They were divided into test groups consisting of five or six animals.

At least two preimmunization C' and C'4 titers were taken at bleeding intervals of four days so that comparisons could be made with postimmunization titers. If variations were found in the data of the first two titrations, subsequent measurements were made until two titrations in succession showed practically the same results. The titers were then averaged to establish the preimmunization titer of each animal.

The antigens to be tested were divided into three groups, bovine blood serum fractions, antigens of unrelated origin, and Staphylococcus aureus vaccine. All antigens were prepared with 0.85 per cent sterile saline. All except the vaccine were diluted to a concentration of five per cent, and each was preserved with phenol in a final concentration of 0.5 per cent. The S. aureus vaccine was prepared from a 24 hour broth culture. After centrifugation and removal of the supernate, the cells were washed with sterile 0.85 per cent saline, and then centrifuged. The supernate was again removed. Sterile saline was then added until approximately a suspension of one to three billion per milliliter cells was obtained. This suspension was kept for stock and subsequently diluted to contain one to three million cells per milliliter for injection. The photometric method of Todd, Sanford, and Wells (4) for determination of the number of cells per milliliter was followed. All antigens were injected subcutaneously on the abdomen in 0.5 milliliter amounts.

After standardization of the guinea pigs for C' and C'4 titers three antigens for each group were selected except for the groups which were to be repeatedly immunized

with Staphylococcus aureus vaccine. The first antigen of the series was injected every other day for a total of three injections. Two weeks after the first injection the animals were bled and titered. Four days later they were titered again and the average was determined. The same animals were then injected with the second antigen of the series. The previous injection and titration procedures were followed. The third antigen of the series was then injected following the same schedule. The two groups of animals that were immunized with S. aureus vaccine were injected and titered every four days until all the animals expired.

Five groups of guinea pigs received a series of injections of three bovine blood serum fractions. The first group was injected with albumen, fibrinogen, and gamma globulin. Into the second group of animals was injected albumen, alpha globulin, and gamma globulin. The third group was immunized with albumen, a mixture of albumen and alpha globulin, and alpha globulin. In group four the animals were injected with albumen, a mixture of albumen and beta globulin, and beta globulin. The fifth group received injections of beta globulin, gamma globulin, and albumen.

The group of animals that received injections of antigens of unrelated origin were immunized with Staphylococcus aureus vaccine, ovalbumen, and bovine alpha globulin. After this series was completed the same animals were again injected with the initial antigen, S. aureus vaccine. The original injection and titration schedules were followed. Upon the injection of ovalbumen, however, all animals expired. Signs of analphylaxis such as rapid breathing, coughing, and ruffled hair were noted.

All blood specimens for titration were taken by cardiac puncture from unanaesthetized animals. In general 1.5 to 2 milliliters of blood was taken from each animal and transferred from the syringe directly into a clean test tube and placed in a refrigerator at five degrees C for no less than 30 minutes to allow the sample to clot and to retard degradation of the individual components of complement. Each of the blood samples was then centrifuged and the serum removed with a serological pipette and bulb.

Titration of Total Complement and C'4

An aliquot of 0.15 milliliter portion of serum was taken from the samples from each guinea pig for titration of the C'4 fraction. A 0.1 milliliter portion was also

taken from each sample for titration of total complement and C'4.

To each 0.15 milliliter portion of serum was added 0.45 milliliter of 0.12 normal ammonium hydroxide. The mixture was allowed to stand at room temperature for one and one-half hours. This process inactivated C'4, and left C'1, C'2, and C'3 active. At the end of the inactivation period 0.45 milliliter of 0.12 normal hydrochloric acid was added to each aliquot to neutralize. The serum was then diluted to 1:100 or 1:200 with a buffered saline solution containing 8.5 grams of C. P. sodium chloride in demineralized water. One milliliter of stock buffer was added to the saline. This stock buffer contained 7.5 grams of magnesium chloride and 2.5 grams of calcium carbonate in 100 milliliters of demineralized water. All titrations were made using the buffered saline. Three 3.5 milliliter aliquots of this serum containing inactivated C'4 was then added to three samples of untreated serum and their final volume of untreated serum was adjusted to 1:10,000, 1:15,000, and 1:20,000.

Hemolysin (anti-sheep red blood cell serum from the rabbit suspended in glycerine) was diluted to a 1:10,000

concentration. Cappel sheep red blood cells were washed with saline until the supernate was clear and then enough packed cells were added to the hemolysin dilution to obtain a five per cent concentration of cells. After this treatment, the sensitized cells were incubated for 10 minutes at 37 degrees C and then stored in the refrigerator until use.

In the tests using a 1:100 dilution of serum containing inactivated C'4 the saline, untreated serum, serum with inactivated C'4, and sensitized sheep red blood cells were pipetted into test tubes as shown in the following table.

TABLE I

PROCEDURE FOR TEST TUBE DILUTIONS

Tube No.	Saline	Normal Serum	Treated Serum	Sensitized Red Blood Cells
1	3.0 ml	1.0 ml of 1:100	. .	1.0 ml
2	3.0 ml	1.0 ml of 1:400	. .	1.0 ml
3	0.25 ml	0.25 ml of 1:10,000	3.5 ml	1.0 ml
4	0.25 ml	0.25 ml of 1:15,000	3.5 ml	1.0 ml
5	0.25 ml	0.25 ml of 1:20,000	3.5 ml	1.0 ml
6	4.0 ml	. .	. .	1.0 ml
7	4.0 ml H_2O	. .	. .	1.0 ml
8	3.0 ml	. .	1.0 ml	1.0 ml

The procedure outlined in Table I was also used with the 1:200 dilution of serum containing inactivated C'4. It was necessary to dilute the serum for inactivation of C'4 to 1:200 as a result of the high preimmunization titer that they possessed. The tests in which this dilution was made were two series of injections with bovine blood serum fractions and one series of repeated injections of Staphylococcus aureus vaccine. The blood serum fraction tests were those of injection of six animals with albumen, a mixture of albumen and beta globulin, and beta globulin. Six other animals were immunized with beta globulin, gamma globulin, and albumen.

In tubes one and two the total complement activity is measured. Actually, it has been established that these readings are measurements of the C'2 component since it is the fraction present in least amount (2). Test tubes number three, four, and five yield readings of C'4 activity since this fraction has been made that present in least amount and therefore the limiting factor. Tube six is a negative control and tube seven is a positive control. Tube eight is a control for determination of the effectiveness of inactivation of C'4.

After all ingredients were placed in the various test tubes and mixed thoroughly, they were incubated at 37 degrees C for 45 minutes in a water bath. At the end of this period, they were centrifuged to remove the unlysed cells. The supernate was poured into a cuvette to be read in a Bausch and Lomb Spectronic 20 colorimeter at 5500 Å. The zero adjustment was made using the positive control and the negative control was used to set the 100 per cent transmittance reading. The per cent transmittance recordings were converted into 50 per cent hemolytic units using the conversion tables established by Kabat and Mayer from the Von Krogh equation (3).

Except for changes in dilutions of various reagents, the above titration procedure is the same as that proposed by Kabat and Mayer (3).

Antibody Titrations

In three of the tests antibody titers were also taken along with the complement titration schedule. Precipitin titers were taken with two of the bovine blood serum fraction tests. One group received a series of injections of albumen, an albumen and beta globulin mixture, and beta globulin. The other group was immunized with beta

globulin, gamma globulin, and albumen. Agglutination titers were made on one group of six animals that were being immunized with Staphylococcus aureus vaccine. Serum for these titers was obtained after that for the complement titration had been removed from each tube. Unbuffered 0.85 per cent saline was used. In both tests, standard serial dilution agglutination and precipitation techniques were employed. The titers for each were read after 18 hours. Agglutination tests were incubated in a water bath at 50 degrees C. Precipitin titrations were allowed to remain at room temperature. The titers for both are expressed as the highest serum dilution that yielded positive results.

CHAPTER BIBLIOGRAPHY

1. Boulanger, P., Plummer, P. J. G., Annau, E., and Rice, C. E., "Parallel Studies of Complement and Blood Coagulation XII: The Effects of Pregnancy and Sex Hormones," Cornell Veterinarian, XLIV (1954), 191-198.

2. Carpenter, Philip L., Immunology and Serology, Philadelphia, W. B. Saunders Co., 1956.

3. Kabat, E. D., and Mayer, M. M., Experimental Immunochemistry, Springfield, Illinois, Charles C. Thomas, 1948.

4. Todd, J. C., Sanford, A. H., and Wells, B. B., Chemical Diagnosis by Laboratory Methods, Philadelphia, W. B. Saunders Co., 1953.

CHAPTER III

RESULTS

The data obtained from immunization tests with bovine blood serum fractions are shown in Tables II through VI. Results from immunization with antigens of unrelated origin are found in Table VII. The two series of tests with repeated injections of Staphylococcus aureus vaccine are shown in Tables VIII and IX. The results in all tables are average values of total complement and C'4 titers. These are expressed in 50 per cent hemolytic units taken from Kabat and Mayer (2). With the exception of the tests with the S. aureus vaccine, the tables are arranged in columns showing data for each animal. The average preimmunization and postimmunization titers following each antigen injection series are given. In Tables V, VI, and IX antibody titers are also presented. In Table IX average C', C'4, and agglutination titers at four-day intervals are recorded. Each animal is not shown separately.

Table II shows the C' and C'4 titers following immunization with albumen, fibrinogen, and gamma globulin.

TABLE II

C' AND C'4 TITERS* IN GUINEA PIG SERUM FOLLOWING IMMUNIZATION WITH ALBUMEN, FIBRINOGEN, AND GAMMA GLOBULIN**

Animal	Pre-Immunization		Postimmunization					
			Albumen		Fibrinogen		Gamma Globulin	
	C'	C'4	C'	C'4	C'	C'4	C'	C'4
1	256	10,665	324	14,130	227	10,130	366	12,037
2	256	10,665	408	14,707	328	10,440	378	12,135
3	304	10,264	328	14,910	336	9,660	. .	. . .
4	256	10,854	324	15,120	256	9,660	. .	. . .

*Titers are expressed in 50 per cent Hemolytic units.

**Albumen was injected on 1/30, 2/1, and 2/3; the titrations were on 2/19 and 2/23. Fibrinogen was injected on 2/23, 2/25, and 2/27; the titrations were on 3/18, 3/22, and 3/26. Gamma globulin was injected on 3/26, 3/28, and 3/30; the titrations were on 4/9 and 4/13.

An increase in the C'4 titer is seen after immunization with albumen. This response was expected as the result of investigations by numerous workers who have reported a general increase in C'4 following immunization with most antigenic substances. This has been recently investigated by Guthrie (1). The increase in C'4 titer is seen to be more than 3,500 hemolytic units in all animals. Only four

animals are shown in Table II as two expired after one titration following albumen immunization. When these animals were injected with fibrinogen, there was a reduction in the titer which, as can be seen in subsequent charts, was characteristic of the C'4 titer after immunization with the second in a series of bovine blood serum fraction injections actually fell below that of the preimmunization titer, the greatest decrease being 1,194 units. However, animals three and four have units of 9,660 which is the lower limit of C'4 which could be titered at the serum dilution used in the test. This is explained by Kabat and Mayer (2) as being due to the inaccuracy of the colorimeter above 92 per cent transmittance and the non-logarithmic relationship between the amount of C'4 and the number of red blood cells lysed above this reading. Following immunization with gamma globulin only two titers of C' and C'4 are shown. Animals three and four expired during the second bleeding for the previous set of titers. It is noted that titers of both animals increased after this series of injections; however, the increase did not reach the level of titers after the first antigen injection.

The effect of repeated injections of bovine albumen, alpha globulin, and gamma globulin are found in Table III.

TABLE III

C' AND C'4 TITERS* IN GUINEA PIG SERUM FOLLOWING IMMUNIZATION WITH ALBUMEN, ALPHA GLOBULIN, AND GAMMA GLOBULIN**

Animal	Pre-Immunization		Postimmunization					
			Albumen		∝ Globulin		ϒ Globulin	
	C'	C'4	C'	C'4	C'	C'4	C'	C'4
1	256	17,015	256	19,800	256	12,660	256	22,425
2	272	19,430	256	19,800	256	12,660	256	21,540
3	264	18,559	256	17,994	256	15,000	256	21,540
4	256	17,378	256	19,800	256	12,660	256	22,069
5	256	14,708	256	17,835	256	14,415	256	21,540
6	256	14,995	256	17,152	. .	. . .	. .	. . .

*Titers are expressed in 50 per cent hemolytic units.

**Albumen was injected on 6/29, 7/1, and 7/3; titrations were on 7/13 and 7/18. Alpha globulin was injected on 7/18, 7/20, and 7/22; titrations were on 8/16, 8/20, and 8/24. Gamma globulin was injected on 8/24, 8/26, and 8/28; titrations were on 9/9 and 9/13.

The preimmunization C'4 titers in this group of animals were considerably higher than those of most other tests. However, an increase in titer following injection of albumen was demonstrated. Upon injection of alpha globulin, the

C'4 activity diminished considerably, showing the same pattern as the group of animals in Table II. The measured maximum decrease of 7,140 hemolytic units was much greater than the observed decrease after the second antigen was injected in the test shown in Table II. There was also a large increase in the titer of C'4 following the injection series of gamma globulin. Unlike the response shown in Table II to gamma globulin, the titers in Table III following gamma globulin immunization were greater than those established after the first antigen was injected. Animal number six expired during the second bleeding following the first antigen injection.

The C' and C'4 titers after immunization with albumen, a mixture of alpha globulin and albumen, and alpha globulin are shown in Table IV. The preinjection titers of this group of animals are also high. However, an increase in the C'4 titer following immunization with albumen was demonstrated, the greatest being 5,477 hemolytic units. When the same guinea pigs were immunized with a mixture of albumen and alpha globulin, the trend of a depression in the C'4 titer can again be seen. The decrease is not as large as in other tests, but this could be the result of the inclusion of the first antigen in the mixture

injected in the second series. In the third series of immunizations no significant change in the titer can be seen as noted in Table IV.

TABLE IV

C'AND C'4 TITERS* IN GUINEA PIG SERUM FOLLOWING IMMUNIZATION WITH ALBUMEN, ALBUMEN AND ALPHA GLOBULIN, AND ALPHA GLOBULIN**

Animal	Pre-Immunization		Postimmunization					
			Albumen		Albumen and α Globulin		α Globulin	
	C'	C'4	C'	C'4	C'	C'4	C'	C'4
1	256	18,690	256	21,690	288	21,540	256	19,245
2	256	18,690	256	23,280	203	20,522	256	19,800
3	256	20,985	256	21,705	256	20,522	256	20,520
4	256	16,980	256	22,357	288	21,540	256	21,570
5	256	18,690	256	21,504	344	22,609	256	21,385
6	256	19,800	256	23,280	. .	. . .	. .	. . .

*Titers are expressed in 50 per cent hemolytic units.

**Albumen was injected on 8/29, 8/31, and 9/2; titrations were on 9/14, 9/18, and 9/22. Albumen and alpha globulin were injected on 9/25, 9/27, and 9/29; titrations were on 10/10 and 10/14. Alpha globulin was injected on 10/23, 10/25, and 10/27; titrations were on 11/6 and 11/10.

In Table V are shown C', C'4, and precipitin titers following immunization with albumen, a mixture of albumen and beta globulin, and beta globulin. The preimmunization C'4 titers are low. A 1:200 dilution of serum containing inactivated C'4 was used instead of a 1:100 dilution. All animals show an elevated level of C'4 activity after albumen immunization. Although increases appear significant, it is suspected that larger increases than indicated occurred since is is probable that the actual preimmunization titers were lower than shown. The precipitin titers after albumen immunization, as in all tests, are expressed as the highest serum dilution which yielded precipitation. All animals are shown to have had an immune response to albumen as indicated by their precipitin titers. A slight decrease in the C'4 activity after immunization with a mixture of albumen and beta globulin is noted. The precipitin titer for this series of immunizations show that the ability to have an immune antibody response did not decrease. No significant changes are seen in the C'4 titer after beta globulin was injected. The precipitin titer for beta globulin is, however, higher than that for either of the previous injections. These data are included in Table V, page 27.

TABLE V

C', C'4,* AND PRECIPITIN TITERS** IN GUINEA PIG SERUM FOLLOWING IMMUNIZATION*** WITH ALBUMEN, ALBUMEN AND BETA GLOBULIN, AND BETA GLOBULIN

Animal	Pre-Immunization		Postimmunization								
			Albumen			Albumen and β Globulin			β Globulin		
	C'	C'4	C'	C'4	Precipitin	C'	C'4	Precipitin	C'	C'4	Precipitin
1	256	9,660	256	10,065	1:40	256	10,653	1:120	256	10,440	1:160
2	256	9,660	256	11,070	1:60	256	9,863	1:120	256	10,065	1:160
3	256	9,660	256	10,968	1:40	256	10,865	1:80	256	10,440	1:160
4	256	9,660	256	10,853	1:60	256	10,856	1:160	256	10,065	1:240
5	256	9,660	256	10,440	1:60	. .	. . .	. .	. .	. . .	. .
6	256	9,660	256	11,708	1:40	256	11,220	1:80	. .	. . .	. .

*Titers are expressed in 50 per cent hemolytic units.

**Average titers.

***Albumen was injected on 3/6, 3/8, 3/10; titrations were on 3/23 and 3/27. Albumen and beta globulin were injected on 3/27, 3/29, and 3/31; titrations were on 4/10 and 4/14. Beta globulin was injected on 4/14, 4/16, and 4/18; titrations were on 4/28 and 5/1.

Table VI, page 29, shows C', C'4, and precipitin titers following immunization with beta globulin, gamma globulin, and albumen. Serum containing inactivated C'4 was diluted 1:200 as in the test shown in Table V. A definite increase in C'4 activity is observed following immunization with beta globulin. The antibody response to this antigen is indicated by the precipitin titer. After the second series of antigen injections, the previously observed suppression of complement activity is noted. However, no inhibition of the antibody response to this antigen is indicated by the precipitin titer. When the third antigen of this series, albumen, was injected, there was again an increase in the C'4 titer, accompanied by the formation of antibodies as shown by the precipitin titer.

C' and C'4 titers following immunization with Staphylococcus aureus vaccine, ovalbumen, and alpha globulin are shown in Table VII, page 31. Upon completion of titrations after the third series of injections, the initial antigen of the series, S. aureus vaccine was again injected. Original injection and titration procedures were followed. Then these animals were again

TABLE VI

C', C'4,* AND PRECIPITIN TITERS** IN GUINEA PIG SERUM FOLLOWING IMMUNIZATION***
WITH BETA GLOBULIN, GAMMA GLOBULIN, AND ALBUMEN

Animal	Pre-Immunization		Postimmunization								
			β Globulin			γ Globulin			Albumen		
	C'	C'4	C'	C'4	Precipitin	C'	C'4	Precipitin	C'	C'4	Precipitin
1	256	9,660	256	10,865	1:80	256	9,660	1:160	256	11,070	1:120
2	256	9,660	256	10,653	1:40	. .	. .	. .	. .	. .	. .
3	256	9,660	256	11,370	1:80	256	9,660	1:80	256	11,220	1:80
4	256	9,660	256	10,968	1:60	256	9,660	1:120	256	11,558	1:80
5	256	9,660	256	11,370	1:40	256	9,660	1:40	256	10,856	1:60

*Titers are expressed in 50 per cent units.

**Average Titers.

***Beta globulin was injected on 3/3, 3/5, and 3/7; titrations were on 3/19 and 3/23. Gamma globulin was injected on 3/23, 3/25, and 3/27; titrations were on 4/6 and 4/10. Albumen was injected on 4/10, 4/12, and 4/14; titrations were on 4/24 and 4/28.

injected with the second antigen of the series, ovalbumen. All animals expired showing symptoms of anaphylaxis.

The titers of C'4 after the first series of injections with Staphylococcus aureus vaccine are greater than the preimmunization titers, the largest increase being 11,420 hemolytic units. The response to the second antigen injected, however, is just the opposite of that observed in the tests with bovine blood serum fractions. In this test the C'4 titers following immunization with ovalbumen did not diminish significantly. On the contrary there was an increase in the titer in animals one, five, and six. However, when this antigen was followed by the injection of a similar antigen, alpha globulin, it can be seen that there was a reduction in the C'4 titer. Upon reinjection of these animals with S. aureus vaccine the previously established response of an increase in titer after immunization with vaccine in animals who had just been injected with a bovine blood serum fraction is not seen. Actually, the opposite result is observed as the titer of C'4 diminished.

In Table VIII are shown C' and C'4 titers in guinea pig serum following immunization at four day intervals with S. aureus vaccine. The number of animals used in

TABLE VII

C' AND C'4 TITERS* IN GUINEA PIG SERUM FOLLOWING IMMUNIZATION WITH STAPHYLOCOCCUS AUREUS VACCINE, OVALBUMEN, AND ALPHA GLOBULIN**

Animal	Pre-Immunization		Postimmunization							
			S. aureus Vaccine		Ovalbumen		∝ Globulin		S. aureus Vaccine	
	C'	C'4	C'	C'4	C'	C'4	C'	C'4	C'	C'4
1	256	11,270	256	19,800	322	23,280	256	16,050	256	16,050
2	264	11,715	256	18,690	256	17,715	256	16,514	300	13,995
3	256	10,837	256	21,570	368	20,110	269	16,385	. .	. .
4	285	11,395	256	22,815	262	21,214	256	15,503	320	12,690
5	256	13,245	256	19,800	256	21,214	256	16,039	. .	. .
6	256	9,660	276	19,246	356	23,280	256	14,817	312	13,492

*Titers are expressed in 50 per cent hemolytic units.

**S. aureus was injected on 4/5, 4/7, and 4/9; titrations were on 4/19 and 4/23. Ovalbumen was injected on 4/23, 4/25, and 4/27; titrations were on 5/8 and 5/12. Alpha globulin was injected on 5/13, 5/15, and 5/17; titrations were on 6/15, 6/19, 6/23, and 6/27. S. aureus was again injected on 6/27, 6/29, and 7/1; titrations were on 7/11 and 7/15.

the test is not constant, however, due to fatalities following bleeding. After the first injection there was an average increase of 3,490 hemolytic units in the C'4 titer. There was also an increase in the average titer after the second injection. This level was maintained for two subsequent injections, but after the fifth injection there was a decrease in the average titer. This was followed by another decrease in C'4 after the sixth injection. Two progressive increases are seen after injections seven and eight, the latter being the maximum average titer reached during the tests. Following this peak, there is noticed a gradual reduction in the C'4 titer until all animals expired. No variations in total complement activity are seen during the entire test. These data are included in Table VIII, page 33.

Table IX, page 35, shows C', C'4, and agglutination titers following repeated injections at four-day intervals with Staphylococcus aureus vaccine. No variations in total complement are seen throughout the test. However, after the first injection an elevation in the C'4 titer is noted. Further increases are seen after the second and third injections, with the maximum titer of the test being seen

TABLE VIII

C' AND C'4 TITERS* IN GUINEA PIG SERUM FOLLOWING IMMUNIZATION AT FOUR-DAY INTERVALS WITH STAPHYLOCOCCUS AUREUS VACCINE

		Average Preimmunization Titer	C' 256	C'4 9,660
Number of Animals	Date of Injection and Titration	Average Titer Following Each Injection		
6	7/28	1	256	13,150
6	8/2	2	256	20,439
5	8/6	3	256	20,207
5	8/10	4	256	20,126
5	8/14	5	256	13,588
5	8/18	6	256	12,889
4	8/22	7	256	18,843
4	8/27	8	256	23,048
4	8/31	9	256	10,854
3	9/4	10	256	17,820
3	9/8	11	256	14,432
2	9/12	12	256	11,020
2	9/16	13	256	10,724

*Titers are expressed in 50 per cent hemolytic units.

after the third injection. The fourth and fifth injections were followed by reductions in the fourth component. This pattern was broken, however, by an elevation in C'4 after the sixth injection. Beginning with the seventh injection a consistent decrease throughout the remainder of the test is noted.

The average agglutination titer does not appear to follow the fluctuating pattern of C'4 activity. As can be seen in Table IX no agglutination titer appeared after the first injection of the vaccine. Following the second injection, however, the antibody response is noted by the 1:28 agglutination titer. Subsequent injections and titrations show a consistent increase in the agglutination titer to a maximum of 1:160. No decrease is noted as in the C'4 titer.

TABLE IX

C', C'4, AND AGGLUTININ TITERS* IN GUINEA PIG SERUM FOLLOWING IMMUNIZATION WITH STAPHYLOCOCCUS AUREUS VACCINE

Average Preimmunization Titer			C' 256	C'4 9,660	Agglutinin 0
Number of Animals	Date of Injection and Titration	Average Titer Following Each Injection			
6	2/28	1	256	10,253	0
5	3/4	2	256	11,446	1:28
5	3/8	3	256	13,194	1:132
4	3/12	4	256	12,960	1:100
4	3/16	5	256	10,516	1:107
3	3/20	6	256	11,237	1:133
3	3/24	7	256	10,130	1:160
3	3/28	8	256	11,370	1:160
3	4/1	9	256	11,102	1:160
3	4/5	10	256	11,370	1:160
3	4/9	11	256	10,932	1:160
3	4/13	12	256	10,440	1:160
3	4/17	13	256	10,190	1:160
2	4/21	14	256	10,065	1:160

*Titers are expressed in 50 per cent hemolytic units.

CHAPTER BIBLIOGRAPHY

1. Guthrie, R. K., Hilton, L. H., Renshaw, L., and Whalen, P. L., "Effect of Antigen Injection on the Fourth Component of Complement," The Texas Journal of Science, XII (May, 1960), 55-60.

2. Kabat, E. D., and Mayer, M. M., Experimental Immunochemistry, Springfield, Illinois, Charles C. Thomas, 1948.

CHAPTER IV

DISCUSSION

The data obtained in this investigation show that after the first injection series of all tested antigens there was an increase in the C'4 component of complement but not in total complement. These findings support previous reports of Guthrie (2). Different responses were noticed following the injections of a second type of antigen. In tests with blood serum fractions the second series of immunizations was followed by a decrease in the C'4 titer, even when the injection contained a mixture of the previously tested blood serum fraction plus an additional one. In this latter test the third series of antigen injections included only the new antigen introduced in the mixture of the second series, and no change in the C'4 titer was observed. However, if the third series of immunizations did not contain a previously injected antigen the animal's response was that of an increase in the C'4 titer or the titer remained at

the level of the previous test. No depression in the titer was noted.

In the tests where the first two antigen injections were of different types, such as Staphylococcus aureus and ovalbumen, the response to the second antigen was opposite that obtained in tests with antigens of related origin. This immunization series was followed by an elevation in C'4 activity. However, when the ovalbumen injections were followed by immunization with bovine alpha globulin, the C'4 titer decreased. Reinjection of these animals with S. aureus vaccine as a fourth injection series did not appear to stimulate further increases in the titer. In fact, one-half of the animals showed a decrease in titer. These animals were then reinjected with ovalbumen as the beginning of a fifth series of injections. After the first injection, however, all expired showing signs of anaphylaxis.

In the tests of repeated immunization and titration at four-day intervals with S. aureus vaccine a curve was indicated showing fluctuations in the C'4 titer which does not follow the usual antibody titer curves. Table VIII shows C'4 titers, while Table IX contains both C'4 and agglutination titers. In both tests similar variations in

the C'4 titer are seen. After the first injection, the C'4 activity is noted to increase. The maximum titer is maintained for short duration and then begins variable decreases. The average antibody titer, however, showed a progressive increase to a maximum level with the exception of titers shown after the fourth and fifth injections. These average titers appear to diminish due to the expiration of a high titered animal. These results agree partially with the findings of Hard (4). He injected bovine serum albumen at regular intervals into rabbits and noticed a rise in the precipitin titer for two months, after which a progressive reduction occurred. However, no decrease in agglutination titers was seen in these tests.

The precipitin titers after each bovine blood serum fraction immunization show that there are individual responses to each antigen. None appear to diminish as did the C'4 titers, especially after the second series of immunizations. This, along with the agglutination titers suggest that the C'4 and antibody responses to an injection of antigen occur independently and do not follow the same titer pattern. Since the antibody titer did not

decrease in the repeated vaccine injections as did the C'4 titer, it appears that either the biosynthesis of C'4 is not as rapid as is antibody formation, or that an exhaustive mechanism in localities of complement synthesis can occur. The methods used in this investigation cannot, however, substantiate this statement.

No significant changes in total complement were observed in any of the tests. This agrees with previous observations by Guthrie (3) and Axelrod (1). Guthrie showed statistically that total complement activity did not increase during immunization but that there were significant changes in C'4. He also showed that these increases were significantly greater than those observed in uninjected animals that were titrated on the same bleeding schedule as experimental animals.

CHAPTER BIBLIOGRAPHY

1. Axelrod, A. E., and Pruzansky, J., "Role of Vitamins in Antibody Production, Vitamins and Hormones, XIII (1955), 14-15.

2. Guthrie, R. K., Hilton, L. H., Renshaw, L., and Whalen, P. L., "Effect of Antigen Injection on the Fourth Component of Complement," The Texas Journal of Science, XII (May, 1960), 55-60.

3. Guthrie, R. K., "A Study of the Relations Between the Blood Antiprotease System and Complement Activity in Specific and Non-Specific Immunological Reactions," unpublished doctoral dissertation, Department of Microbiology, Baylor University, Waco, Texas, 1954.

4. Hard, S., "The Decrease in the Concentration of Precipitation Antibodies in Rabbits after Long-Term Local Antigen Administration, Acta Allergologica, XIII (January, 1959), 1-7.

CHAPTER V

SUMMARY

This investigation has yielded data which indicate that changes occur in the C'4 component of guinea pig complement but not in total complement after repeated antigen injection according to the type of antigen and frequency of injection. Antibody titers were not shown to correspond completely with these changes. All antigens tested show an elevation in the C'4 titer after the first injection or series of injections with the same antigen. The response to the second series of injections was seen to be dependent upon the type of antigen. In tests with similar antigens such as bovine blood serum fractions the titer either remained at the level established after the first immunization series, or it diminished. If, however, the second antigen was not related to the first, the response was that of an increase in the titer. After the third series of related antigen injections, the animals show an increase in titer if the animal had never

contacted the antigen previously. However, if this series included one of the blood serum fractions contained in the second series, no further elevation was noted. If the antigens were unrelated, elevations in the titer were noted after each antigen series. Repeated injections of Staphylococcus aureus vaccine yielded C'4 titers which are not characteristic of normal antibody titration curves. Agglutination titers in this test did, however, follow the standard curve except that the animals expired before exhaustion could be shown.

These investigations suggest that the stimulus for the biosynthesis of C'4 depends upon the type of antigen and the frequency of contact with the animal and that the response is independent of antibody formation. It appears from comparison of antibody and C'4 titers that complement is not formed at the same rate as antibodies, or that exhaustive mechanisms occur in C'4 synthesis. It is also possible that both of these could occur. This investigation suggests but does not prove these conclusions. Further research as to the site of complement and antibody formation along with precise analytical methods should add information to these questions.

BIBLIOGRAPHY

Books

Bordet, J., Resume of Immunity, Studies in Immunity, collected and translated by F. P. Gay, New York, John Wiley and Sons, Inc., 1901.

Carpenter, Philip L., Immunology and Serology, Philadelphia, W. B. Saunders Co., 1956.

Cushing, J. E., Campbell, D. H., Principles of Immunology, New York, McGraw-Hill Co., 1957.

Kabat, E. D., and Mayer, M. M., Experimental Immunochemistry, Springfield, Illinois, Charles C. Thomas, 1948.

Todd, J. C., Sanford, A. H., and Wells, B. B., Chemical Diagnosis by Laboratory Methods, Philadelphia, W. B. Saunders Co., 1953.

Zinsser, H., Enders, J. P., and Fathergill, L. D., Immunity Principles and Applications in Medicine and Public Health, 5th ed., New York, Macmillan Co., 1939.

Articles

Axelrod, A. E., and Pruzansky, J., "Role of Vitamins in Antibody Production," Vitamins and Hormones, XIII (1955), 14-15.

Becker, E. L., "Concerning the Mechanism of Complement Action.V. The Early Steps in Immune Hemolysis," Journal of Immunology, LXXXIII (September, 1960), 299-308.

Boulanger, P., Plummer, P. J. G., Annau, E., and Rice, C. E., "Parallel Studies of Complement and Blood Coagulation XII: The Effects of Pregnancy and Sex Hormones," Cornell Veterinarian, XLIV (1954), 191-198.

Brumfield, H. P., "A Factor of Complement Inactivation by Histamine or Ethylenediamine," Journal of Immunology, LXXII (May, 1954), 393-397.

Dozois, T. F., "The Role of Human Complement in Bactericidal Phenomena," Journal of Immunology, XLVII (March, 1945), 229.

Ecker, E. E., "Complement," Annual Review of Microbiology, II (1948), 121.

Ecker, E. E., Pillemer, L., Jones, C. B., and Seifter, S., 'Separation of Complement from Fresh Guinea Pig Serum," Journal of Biological Chemistry, CXXV (June, 1940), 347.

Guthrie, R. K., Hilton, L. H., Renshaw, L., and Whalen, P. L., "Effect of Antigen Injection on the Fourth Component of Complement," The Texas Journal of Science, XII (May, 1960), 55-60.

Hard, S., "The Decrease in the Concentration of Precipitation Antibodies in Rabbits after Long-Term Local Antigen Administration, Acta Allergologica, XIII (January, 1959), 1-7.

Moore, H. D., "Complementary and Opsonic Functions in Their Relation to Immunity," Journal of Immunology, IV (1919), 425-441.

Muschel, L. H., and Treffers, H. P., "Quantitative Studies on the Bactericidal Actions of Serum and Complement," Journal of Immunology, CIII (January, 1956), 20-27.

Nungester, W. J., "Non-Specific Factors in Immunity," Annual Review of Microbiology, VII (1954), 363-392.

Pillemer, L., and Ecker, E. E., "Anticomplementary Factor in Fresh Yeast," Journal of Biological Chemistry, CXXXVII (January, 1941), 139.

Rice, C. E., Boulanger, P., and Annau, E., "The Effect of Repeated Injections of Dextran or Polyvinylpyrolidone on Serum Complement in the Guinea Pig," Canadian Journal of Public Health, XLV (1954), 264-267.

Unpublished Materials

Guthrie, R. K., "A Study of the Relations Between the Blood Antiprotease System and Complement Activity in Specific and Non-Specific Immunological Reactions," unpublished doctoral dissertation, Department of Microbiology, Baylor University, Waco, Texas, 1954.

Schlagenhauf, G. K., "A Study of the Role of Complement in the Protection of AKr Mice Against Transplanted Lymphoid Leukemia," unpublished master's thesis, Department of Biology, North Texas State College, Denton, Texas, 1957.