RELATION OF DOSAGE OF ANTISERUM TO

PROTECTION IN MOUSE LEUKEMIA

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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

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Denton, Texas

January, 1961

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CHAPTER I

INTRODUCTION

Recent evidence has been presented which indicates that more factors are involved in the immune process than are usually considered. The factor which seems to be of most importance is an activity which is present in the serum of almost all of the warm-blooded animals which is now called complement. The first observations of this activity were recorded in the late nineteenth century by such workers as Buchner, Van Fordor, Nuttall, Bordet, and Pfeiffer (9). Buchner, Van Fordor, and Nuttall demonstrated a destructive influence of blood serum on bacteria. Bordet demonstrated in vitro bacteriolysis and observed that the action could be destroyed by heating the immune serum to 56 degrees Centigrade. The addition of normal serum to the heat-inactivated serum restored the action. This indicated that two distinct substances were required for bacteriolysis. One of these was easily destroyed by heat and was present in normal serum. The other was more stable and was produced or increased as a result of immunization. Pfeiffer demonstrated in vivo complement activity when he reported the lysis of cholera vibrios when injected into the peritoneum of guinea pigs

which had recovered from experimental cholera infections. This action could not be demonstrated in normal animals and indicated that it was due to a specific immune reaction. This action could be transferred to normal animals, however, by intraperitoneal injections of either heated or fresh serum from immune animals.

Complement was later found to be composed of several components, and each of these had to be present before complement activity could be demonstrated. The first and second components (C'1 and C'2) were discovered by Ferrata in 1907. He dialysed guinea pig complement against water and separated it into a soluble fraction and an insoluble fraction. Both fractions were devoid of hemolytic activity while separated, but recombination of the two restored their activity. The third and fourth components (C'3 and C'4) were discovered by the demonstration that complement activity was destroyed by treatment with cobra venom, yeast, or zymosan. A factor other than C'1 and C'2 was inactivated. This factor, designated as C'3, was relatively heat-stabile in contrast to C'1 and C'2 which are very sensitive to heat. The fourth component (C'4) was also thermostabile. It was recognized by the fact that complement activity could be destroyed by treatment with ammonia. The dialysis of complement actually separates the four components into two groups. Most of the C'3 remains with C'1 whereas the C'4 is soluble and most of it is found

with the C'2. Based on the preceding information, at least four components are now recognized with the possibility of more being present.

The lytic action of complement when it is tied up in an antigen-antibody complex has raised many questions as to the role of complement in the immunizing process. Many workers have shown that complement level remains approximately constant during immunization (18). But it has also been shown that during an infection the total complement activity may show an increase. Guthrie (6) reported a small but consistent increase in total complement activity in guinea pigs following immunization with some bacterial antigens. Pillemer (17) also noticed slight rises in total complement activity which he denoted as a non-specific role in the immunizing process. Muschel and Treffers (15), using Salmonella typhosa, surmised that a smaller volume of immune serum could be used to kill as effectively as a larger portion of normal serum.

Hilton (7), working with various components of complement, found higher titers in guinea pigs after injection of antigens and in guinea pigs suffering chronic infections. He found an increased titer in both C'3 and C'4 components with the greatest increase found in C'4. This substantiated the work of Guthrie (6), who observed unusual activity in the fourth component of complement in specific and non-specific immune reactions. Whalen (24) further extended

the work of Guthrie and Hilton by showing a measurable increase in C'4 titer of young rats following injections of egg albumin and bacterial antigens. The C'3 did not show any increase in titer. The injection of non-antigenic materials gave no C'4 increase. Renshaw (19) was able to demonstrate an increase in C' , C'3, and C'4 titers in guinea pigs following injections of egg white. He related the animal's immune responses and the fourth component of complement by noting that cortisone had no effect on the C'4 titer of the animal until it had been antigenically stimulated. This supported previous work which indicated the depressive effect hormones, especially cortisone, have on antibody production (12, 13).

With this indication of a relation of complement and the immune response of animals to certain antigens, experiments were designed to determine the extent of the role of complement and of the various components. Work was done to determine the role of complement in relation to bacterial infections. It was shown (25) that complement was instrumental in lysis of gram-negative organisms. Work by Moore (14) indicated that immune guinea pig serum was effective in increasing the percentage survival of Staphylococcus aureus infected mice. This increase was noted in untreated immune serum, C'1 and C'2 inactivated immune serum, and C'1, C'2, C'3 inactivated immune serum, indicating the C'4 to be the fraction responsible for the

increase. This agreed with Schlagenhauf (20), who had shown that the C'4 component of complement was important in the protective action of immune antiserum against transplanted lymphoid mouse leukemia.

Some work was done prior to that of Schlagenhauf in the field of immunizing against mouse leukemia and other types of cancer. Werder, Kirschbaum, and Syverton (22) in 1950 demonstrated in vitro inactivation of mouse leukemic cells with unheated leukemic tissue rabbit antiserum so that the capacity to transmit the disease was completely inhibited. Serum heated for thirty minutes at 60 degrees Centigrade as well as normal rabbit serum showed only slight inhibitory action. The same workers substantiated this work in a later paper (23) but also included an inhibitory effect of normal rabbit serum. They surmised that there was a heat-labile inhibitor in normal rabbit serum which resulted in survival of some mice in spite of heating. They attributed this inhibition and protection to antibodies. Green (5) also found rabbit antiserum to be inhibitory in vitro for mouse mammary carcinoma. Kidd (11) was able to show regression of two kinds of subcutaneous lymphomas following the injection of normal guinea pig serum into infected mice. He also observed that a single injection of guinea pig serum in mice shortly after tumor implantation usually resulted in the failure of a palpable growth. A second injection always inhibited

tumor growth. Nungester and Fisher (16) were able to demonstrate an antibody directed against some component of the tumor cells. The red blood cell agglutinin present in antitumor sera was ineffective, as well as antinormal tissue sera, for *in vivo* inactivation of the tumor cells.

Kauffman and Kidd (10) noted that lymphoma 6C3HED cells promptly began to die after injection of normal guinea pig serum into infected mice. They indicated host participation due to isoantibodies or some component of complement. *In vitro* inactivation of the lymphoma cells was not demonstrated. Goldstein and Myrick (4) described the cytopathogenic effect of anti-Hela cell rabbit serum which occurred only in the presence of added complement. Colter, Defendi, Wallace, and Bird (1) were unable to show cytotoxicity of normal rabbit serum against human bone marrow leukemia. Rabbit sera from animals immunized with nucleoproteins from the leukemia bone marrow were shown to be cytotoxic. This cytotoxicity was found to be complement dependent and was lost on heating. Schrek and Preston (21) also demonstrated *in vitro* that sera from rats with regressed lymphosarcomas were cytotoxic to the lymphosarcoma cells. This cytotoxicity was not found in normal rat serum, and the action of the immune serum was complement dependent.

The preceding evidence seems to point out the important role of complement in the response of an animal to an

antigenic stimulation as well as the possibility of making use of serum showing an increased C'4 level in the treatment of tumor infected animals. This possible use is further strengthened by the work of Schlagenhauf (20), who showed an increase in the survival time of Akr mice infected with a strain of mouse leukemia following a single injection of immune guinea pig serum. Further work along this line was reported by Elliot (3). In this work evidence was presented which supported earlier work by Renshaw (19) on the effect of cortisone on complement titers. Elliot reported that cortisone injections alone caused a decrease in C'4 titer of guinea pigs. This serum afforded no protection to mice infected with leukemia. The same was true of serum from animals which received both cortisone and tumor antigen. In 1959, Ingebrigsten (8) was able to show that antibodies were not responsible for protection of mice infected with a strain of leukemia. He demonstrated an increased survival average for infected mice which had received injections of immune guinea pig serum from which the specific antibodies had been removed by adsorption. The serum used always had a high C'4 titer. This indicates that the C'4 plays a more important role than the antibody produced as a result of immunization. This work was partially supported by Dunkerly (2), using rabbit and mouse antiserum. There was no apparent increase in either C' or C'4 following injection of the tumor suspension in

rabbits or non-susceptible mice. This serum then gave no protection to the infected susceptible strain of mice.

The reports mentioned seem to indicate the important role of C'4 in the protection of leukemia infected mice. In all of the experiments, a single injection of antiserum was used. It is the purpose of this paper to attempt to confirm the work of Schlagenhauf (20) and Ingebrigsten (8) and to show the effects of repeated injections of guinea pig serum immunized with various strains of mouse leukemia. An attempt is also made at in vitro inactivation of the tumor cells by using normal antiserum and antibody-free antiserum. And last, an attempt is made to show regression of the tumor after it has become palpable by using treated guinea pig immune serum and normal immune serum.

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CHAPTER II

MATERIALS AND METHODS

Test Animals

Guinea pigs were used for the production of immune serum, and strains of inbred mice were selected according to their susceptibility to specific strains of lymphoid leukemia. All of the animals used were young animals, and group members were chosen regardless of sex. Animals were housed in a centrally controlled temperature environment. Guinea pigs were fed a commercial guinea pig chow supplemented with oats, and the mice were fed a commercial mouse ration.

Three strains of mice were used, Akr, AK02F1, and the C57BL6j.

Tumor Strains

Two different strains of tumor were used. The experiment was originally designed using the BW5146 tumor in Akr mice. This was expanded to the AKD2F1 mouse susceptible to the same tumor strain and later to the C1498 tumor strain for which the C57BL6j mice were used. The tumors were obtained from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. The tumor was kept available by successive

transfers of tissue containing the viable tumor by subcutaneous injections of the tumor-cell suspension. Transfer of the tumor consisted of removal of the infected spleen, lymph nodes, and the tumor tissue at the inguinal site of injection. This tissue was placed in a tissue homogenizer and diluted with a sterile, buffered saline medium (Gey Solution). The BW5146 tumor was transferable fourteen days after injection, and the C1498 tumor was transferable eight to ten days after injection.

Complement Titration

The guinea pigs were bled and complement titers determined in order to get a standard complement titer. The titration procedure used was a modification of the method described by Kabat and Mayer (1). About two milliliters of blood for the titrations were obtained by cardiac puncture. The blood was allowed to clot at refrigerator temperature for thirty minutes and the serum removed from the clot. The serum was then diluted 1:100 and 1:400 for a total complement (C') level and 1:10,000, 1:15,000, and 1:20,000 for C'4 titer. Inactivation of C'4 was achieved by the addition of 0.45 milliliters of 0,12 normal ammonium hydroxide. This was incubated at room temperature for one and one-half hours, and the solution was neutralized by the addition of 0.45 milliliters of 0.12 normal hydrochloric acid.

The indicator used was sheep red blood cells which were sensitized with rabbit, anti-sheep cell hemolysin. The cells were washed clean with normal saline, and a predetermined amount of hemolysin added. Incubation of the sensitized cells was at 37 degrees Centigrade for a period of ten minutes.

The final test consisted of six tubes (Table I), which were incubated at 37 degrees Centigrade for forty-five minutes. A positive control consisting of one milliliter

TABLE I

COMPLEMENT TITRATION PROCEDURE

Tube Number	Saline (ml)	Normal Serum (ml)	Inactivated Serum (ml)	RBC+Hemolysin (ml)
1	3.0	1.0 of 1:100		1
2	3.0	1.0 of 1:4000		1
3	0.25	0.25 of 1:10,000	3.5 of C'4	1
4	0.25	0.25 of 1:15,000	3.5 of C'4	1
5	0.25	0.25 of 1:20,000	3.5 of C'4	1
6	3.0		1.0 of C'4	1

of red blood cells plus four milliliters of water and a negative control containing one milliliter red blood cells plus four milliliters of saline were included with the tests. Readings were taken in percentage transmittance on a Bausch and Lomb Spectronic 20 Colorimeter at a wavelength of

5500 Å. The readings were converted into 50 per cent hemolytic units according to a conversion table from Kabat and Mayer (1).

After a suitable standard titer was obtained, the guinea pigs were immunized, and titrations were done to determine any change in the C' and C'4 level of the blood.

Immunization Schedules

The guinea pigs were immunized by a series of three subcutaneous injections of 0.4 milliliters of tumor suspension which had been frozen for several days. The injections were made at forty-eight hour intervals. The guinea pigs were standardized according to the procedure given before injection of the tumor and were titered following the tumor injection. Post-injection titers were determined two weeks after the first tumor injection, and at least two titrations were recorded to insure a standard post-injection titer for C' and C'4.

A total of eighteen guinea pigs were immunized with BW5146 strain tumor, and thirteen guinea pigs were immunized with C1498 strain tumor.

Protection Tests

Several experiments were designed to determine what effect immune guinea pig serum would have on prolongation of life span of lymphoid leukemia infected mice. The serum for these tests was obtained by bleeding the immunized

animals by cardiac puncture and allowing the blood to clot at refrigerator temperature with subsequent removal of the serum from the clot. The serum was divided into two portions. One was left untreated as normal immune serum. To the second portion was added an amount (0.1-0.2 milliliters) of the previously frozen tumor suspension. This was allowed to incubate at refrigerator temperature for two hours to allow adsorption of the antibody molecules onto the tissue particles. In almost all instances the first serum injection and tumor infecting injections were done at the same time.

Experiment 1 was set up to determine the effect of a single injection of antiserum. The test consisted of ten mice injected with normal immune serum, fifteen injected with the adsorbed serum, and ten controls which received no antiserum. All of the animals were given an initial tumor injection of 0.6 milliliter tumor suspension which was followed in the experimental animals by a 0.3 milliliter injection of either the adsorbed or normal antiserum. The tumor strain used was the BW5146 in Akr mice.

Experiment 2 included the use of ten Akr mice which received 0.4 milliliters of untreated antiserum, eight which received 0.4 milliliters of adsorbed antiserum, and seven controls. The serum injections were repeated every forty-eight hours for three injections. The amount of

tumor (BW5146) injected was 0.2 milliliters in both experimental groups and in the controls.

In Experiment 3 the serum injections were repeated every twenty-four hours for seven injections. Each serum injection was 0.4 milliliters in both the adsorbed experimental group and the normal antiserum group. The experimentals and the controls received an initial 0.2 milliliters of tumor suspension simultaneously with first serum injection. In this experiment AKD2F1 mice were used with the BW5146 tumor.

The fourth test involved repeating the antiserum injections (0.4 milliliters) every twenty-four hours until the death of the animal. The mouse strain was Akr and the tumor BW5146. Experimental and control animals were infected with 0.2 milliliters of the tumor suspension at the time of the first antiserum injection. The experimental animals consisted of one group receiving the normal untreated antiserum and one group receiving the adsorbed antiserum.

Test Number 5 used the C1498 tumor strain in C57BL6j mice. The test was set up to use repeated injections of both the treated and normal antiserum until death of the animals. After thirty days one mouse in the adsorbed group was still alive, and the serum injections were abandoned at the end of this thirty-day period. Again 0.4 milliliters

of the serum for the two test groups were used with all of the animals receiving 0.2 milliliters of the tumor suspension.

The sixth test was varied to determine whether or not a palpable tumor could be regressed by use of the adsorbed antiserum or normal antiserum. For this experiment the mice received the 0.2 milliliters of tumor, but the antiserum injections were delayed for seven days. By this time a palpable growth was evident and the antiserum injections (0.4 milliliters) were begun. Injections were made every twenty-four hours until death of the animal. The BW5146 tumor and Akr mouse were used.

A last test was set up to determine in vitro inactivation of the tumor. The viable tumor suspension was mixed with normal antiserum and previously adsorbed antiserum. The antiserum to tumor ratio was two to one. The two mixtures were then injected into the respective test groups. The controls received 0.2 milliliters of the tumor only. No additional injections of serum, mixture of serum plus tumor, or tumor were made.

The procedures of adsorption of the antiserum by antigen and the injection schedules are modifications of those used by Schlagenhauf (3) and Ingebrigsten (2).

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CHAPTER III

RESULTS

The first group of guinea pigs were immunized with BW5146 tumor. Complement titers before and after this immunization are shown in Table II. A total of eighteen animals was included in this group. Of the eighteen animals, fourteen showed an increase in the C' titers following immunization with the BW5146 tumor tissue. The mean C' titer showed an increase from a pre-injection mean of 164 units to a post-injection mean of 221 units, for a mean increase of 57 units. The C'4 group mean increased from 11,139 units to 13,118 units, for an increase of 1,979 units. Three of the animals showed a decrease in C'4 titer. Sera from these animals were used in the Akr and AK02F1 mouse protection tests.

The C' and C'4 titers of the guinea pigs in group two are listed in Table III. Thirteen animals were immunized with C1498 tumor tissue. All of these animals demonstrated a significant C' titer increase and C'4 titer increase following immunization. The mean C' titer showed an increase from 157 units to 276 units, an increase of 119 units. The mean C'4 titer increased from 10,145

TABLE II

C' AND C'4 TITERS IN GUINEA PIG SERUM PRIOR TO AND FOLLOWING IMMUNIZATION WITH BW5146 TUMOR

Animal	Date First Titration-Last Titration	50 Per Cent Hemolytic Units			
		Pre-Immunization		Post-Immunization	
		C'	C'4	C'	C'4
1	1/26-6/6-60	142	9,660	306	13,912
2	3/8-8/3-60	133	9,660	274	13,001
3	2/12-6/6-60	138	10,882	289	12,318
4	2/13-6/6-60	141	9,660	241	10,455
5	3/6-6/6-60	135	9,660	270	11,169
6	2/16-6/6-60	145	9,937	187	10,327
7	2/17-6/6-60	138	9,660	292	12,360
8	12/14-59--4/29-60	166	13,628	193	13,308
9	12/14-59--4/29-60	173	13,751	214	12,129
10	6/25-8/1-60	217	12,962	199	14,200
11	6/25-8/1-60	181	15,837	175	14,194
12	6/25-8/3-60	180	14,534	205	14,912
13	6/25-8/3-60	198	11,396	230	17,996
27	9/9-10/6-60	170	9,660	158	16,970
28	9/9-10/6-60	166	10,567	189	11,449
29	9/9-10/6-60	194	9,660	203	11,436
30	9/9-10/6-60	171	9,660	186	15,629
31	9/9-10/6-60	163	9,728	158	10,361

TABLE III

C' AND C'4 TITERS IN GUINEA PIG SERUM PRIOR TO AND FOLLOWING IMMUNIZATION WITH C1498 TUMOR

Animal	Date First Titration-Last Titration	50 Per Cent Hemolytic Units			
		Pre-Immunization		Post-Immunization	
		C'	C'4	C'	C'4
14	7/16-8/16-60	162	11,224	359	16,896
15	7/16-8/16-60	170	10,256	347	19,388
16	7/16-8/16-60	170	9,660	391	15,572
17	7/16-8/16-60	161	10,616	344	17,929
18	7/16-8/16-60	164	11,908	348	15,434
19	7/16-8/16-60	159	9,939	386	10,075
20	7/18-8/18-60	141	9,660	205	12,107
21	7/18-8/18-60	139	10,120	205	13,781
22	7/18-8/18-60	137	9,660	205	16,300
23	7/18-8/18-60	137	9,660	205	15,327
24	7/22-8/18-60	142	9,660	182	12,232
25	7/22-8/18-60	175	9,862	205	14,299
26	7/22-8/18-60	181	9,660	205	14,881

units to 15,632 units, a mean increase of 5,487 units. The sera from these animals were used in the C57BL6j mouse protection tests.

The first mouse protection tests involved only a single injection of either the normal antiserum or the adsorbed antiserum. Table IV shows the combined results of three of these experiments. A total of thirty-five Akr mice received an initial injection of 0.3 milliliter of the BW5146 tumor suspension. Ten of these thirty-five mice received 0.6 milliliter of normal antiserum, and fifteen

TABLE IV

RESULTS OF A SINGLE ANTISERUM INJECTION ON SURVIVAL OF BW5146 LEUKEMIA INFECTED Akr MICE

Type of Injections	Number of Mice Used	Day of Death						Survival Average (Days)
		13	15	17	18	19	22	
Group One*	11				8	2	1	20.0
Group Two**	10	1		1	5	2	1	18.0
Group Three***	15		2		6	7		18.0

*Mice receiving 0.3 ml. of tumor suspension only.

**Mice receiving 0.3 ml. of tumor suspension and 0.6 ml. of normal antiserum.

***Mice receiving 0.3 ml. of tumor suspension and 0.6 ml. of adsorbed antiserum.

received 0.6 milliliter of the antiserum which had been incubated at refrigerator temperature for two hours with tumor cell debris from frozen tumor suspension. The remaining ten mice served as controls and were injected with tumor only.

The results show the control group to have the longest survival average of twenty days. Both test groups had a survival average of eighteen days. Apparently no protection was given by the single injection of either the normal or adsorbed antiserum. The serum used was from a pool of guinea pigs one and two, both of which showed an increased C' and C'4 titer following immunization, and eight and nine, both of which showed a decrease in C'4 titer.

Table V shows the results of repeated antiserum injections on BW5146 tumor infected Akr mice. Each of the total

TABLE V

RESULTS OF REPEATED ANTISERUM INJECTIONS ON SURVIVAL OF BW5146 INFECTED Akr MICE

Type of Injections	Number of Mice Used	Day of Death					Survival Average (Days)
		13	14	17	18	19	
Group One*	7				6	1	18.1
Group Two**	8	1	2	1	2	2	16.5
Group Three***	8				2	6	18.8

*Mice receiving 0.2 ml. of BW5146 tumor suspension only.

**Mice receiving 0.2 ml. of BW5146 tumor suspension and 3 injections of 0.4 ml. of untreated antiserum.

***Mice receiving 0.2 ml. of BW5146 tumor suspension and 3 injections of 0.4 ml. of adsorbed antiserum.

twenty-three animals used in this test received 0.2 milliliter of the tumor suspension. Guinea pigs number one, two, eight, and nine were again the contributors of the serum used for the test groups. Eight of the mice received injections of 0.4 milliliter of untreated antiserum, and eight received 0.4 milliliter of adsorbed antiserum for a series of three injections given at twenty-four hour intervals. The group receiving the repeated injection of the adsorbed serum had a slightly longer survival average than the control group and a significantly longer survival average than the group receiving untreated serum injections. Group one survival average was 18.1 days, group two was 16.5 days, and group three was 18.8 days.

Table VI shows the results of repeating both types of serum injections every twenty-four hours for seven injections. In this experiment AKD2F1 mice were used, and each of the thirty mice received an injection of 0.2 milliliter of BW5146 tumor suspension. The serum used for this test came from guinea pigs one through seven. All of these animals had shown an increase in C' and C'4 titers. The survival average for each test group had about the same significance as the preceding test. The survival average of group one was eighteen days, of group two was 17.8 days, and of group three was 19.4 days. Again the adsorbed antiserum apparently shows more protection than the untreated antiserum.

TABLE VI

RESULTS OF REPEATING ANTISERUM INJECTIONS FOR SEVEN INJECTIONS ON SURVIVAL AVERAGE OF BW5146 TUMOR INFECTED AKD2F1 MICE

Type of Injections	Number of Mice Used	Day of Death				Survival Average (Days)
		17	18	19	20	
Group One*	5	2	1	2		18.0
Group Two**	12	6	3	2	1	17.8
Group Three***	13	4	7	1	1	19.4

*Mice receiving 0.2 ml. of BW5146 tumor only.

**Mice receiving 0.2 ml. of BW5146 tumor and 0.41 ml. of untreated antiserum.

***Mice receiving 0.2 ml. of BW5146 tumor and 0.4 ml. of adsorbed antiserum.

Results of repetition of the serum injections until the death of the infected mouse are shown in Table VII. Each of the twenty-five Akr mice received the initial 0.2 milliliter of the BW5146 tumor suspension. The mice were divided into two test groups of ten mice per group with five controls. Each test group received 0.4 milliliter of the respective serum every twenty-four hours until the death of the animals. Immune serum was taken from the guinea pigs numbered ten through thirteen. Two of these animals showed a decrease in C' following immunization, but all demonstrated an increase in C'4 titer. The survival average for group one, the control

group, was 16.4 days. Group two had a survival average of 17.1 days and group three, 17.6 days.

TABLE VII

RESULTS OF REPEATED SERUM INJECTIONS UNTIL DEATH OF BW5146 TUMOR INFECTED Akr MICE

Type of Injections	Number of Mice Used	Day of Death							Survival Average (Days)
		13	15	16	17	18	20	21	
Group One*	5			1	3		1		16.4
Group Two**	10		1	3		6			17.1
Group Three***	10	1	2	1	1	1	2	2	17.6

*Mice receiving 0.2 ml. of BW5146 tumor only.

**Mice receiving 0.2 ml. of BW5146 tumor and 0.4 ml. of untreated antiserum.

***Mice receiving 0.2 ml. of BW5146 tumor and 0.4 ml. of adsorbed antiserum.

The results of the tests using C1498 tumor strain in C57BL6j mice are shown in Table VIII. The C1498 tumor is lethal in a much shorter period than the BW5146. These mice received 0.2 milliliter of the tumor suspension and 0.4 milliliter of the antiserum. The antiserum injections were repeated until the death of the individual animal. The serum used was from a serum pool of guinea pigs fourteen through twenty six, all of which demonstrated an increased C' titer and C'4 titer following immunization. The animals

TABLE VIII

RESULTS OF REPEATED ANTISERUM INJECTION OF C1498 TUMOR INFECTED C57BL6j MICE

Type of Injections	Number of Mice Used	Day of Death												Survival Average (Days)
		6	7	8	9	10	15	16	17	18	19	20	30	
Group One*	4			1	1	1		1						10.3
Group Two**	8		2		2	1	1		1		1			9.3
Group Three***	9	1	1	1	2		1			1		1	1	13.9

*Mice receiving 0.2 ml. of C1498 tumor suspension.

**Mice receiving 0.2 ml. of C1498 tumor and 0.4 ml. of normal immune serum.

***Mice receiving 0.2 ml. of C1498 tumor and 0.4 ml. of adsorbed immune serum.

of the control group, group one, showed a survival average of 10.3 days in comparison to 9.3 days for animals receiving repeated injections of untreated antiserum, group two, and 13.9 days for the animals receiving repeated injections of the adsorbed serum, group three. The survival average of group three was increased tremendously by one mouse which survived over thirty days, and serum injections were discontinued at this point. Death actually occurred about ten days later.

Table IX shows the results obtained with delayed injections of antiserum. Each Akr mouse received 0.2 milliliter of tumor suspension. The tumor was palpable after

seven days and the normal immune serum and adsorbed immune serum injections were started at this time. The serum for the injections was obtained from guinea pigs numbered ten through thirteen and twenty seven through thirty one. Three of these showed a decreased C' titer, but all showed an increased C'4 titer following immunization. There was no

TABLE IX

RESULTS OF DELAYED ANTISERUM INJECTION ON BW5146 TUMOR INFECTED Akr MICE

Type of Injections	Number of Mice Used	Day of Death									Survival Average (Days)
		15	17	18	19	20	21	22	23	24	
Group One*	5				2	2	1				19.8
Group Two**	8	1	2		1	1	2	1			19.0
Group Three***	7			1		1		2	1	2	21.9

*Mice receiving 0.2 ml. BW5146 tumor suspension.

**Mice receiving 0.2 ml. BW5146 tumor suspension and 0.4 ml. normal immune serum.

***Mice receiving 0.2 ml. BW5146 tumor suspension and 0.4 ml. adsorbed immune serum.

noticeable regression of the tumor in either the adsorbed serum group or the unadsorbed serum group. The survival averages of the two experimental groups are comparable to previous results in which the serum was injected immediately following the tumor injection. The adsorbed serum group had

a survival average of 21.9 days in relation to nineteen days for the unadsorbed group and 19.8 days for the controls.

The same numbered guinea pigs were bled for the serum in the test for *in vitro* inactivation that were used for the delayed injection test. The results of the *in vitro* tests are shown in Table X. The two experiments were done on the same date, and the controls were the same for both tests. A large number of experimental animals were used for

TABLE X

RESULTS OF *IN VITRO* INACTIVATION OF BW5146 TUMOR BY ADSORBED IMMUNE SERUM AND NORMAL IMMUNE SERUM

Type of Injections	Number of Mice Used	Day of Death						Survival Average (Days)
		19	20	21	27	31	32	
Group One[a]	5	2	2	1				19.8
Group Two[b]	17						1	-----[d]
Group Three[c]	18				1	1		-----[d]

[a]Mice receiving 0.2 ml. BW5146 tumor only.

[b]Mice receiving 0.4 ml. of mixture of normal immune serum and tumor.

[c]Mice receiving 0.4 ml. of mixture of adsorbed immune serum and tumor.

[d]Mice surviving after forty-two days.

this test. Seventeen mice received 0.4 milliliter of the untreated serum plus tumor mixture. Only one of these animals died, and this one lived for thirty-two days. The

group receiving adsorbed serum plus tumor consisted of eighteen mice, and only two of these died before the thirty-second day after injection. These results must be repeated before final conclusions can be reached. The amount of tumor injected in test mice was slightly smaller than the controls received. If the results can be repeated, the test is significant in demonstrating that the adsorbed immune serum is as effective as the unadsorbed immune serum in relation to <u>in vitro</u> inactivation of the tumor.

CHAPTER IV

DISCUSSION

It was not the specific purpose of this paper to study increases in either C' or C'4 titers of guinea pigs following immunization with tumor antigen. It is significant, however, to note that for most of the guinea pigs used in this work (Tables II, III) there was a demonstrable increase in these titers. This increase in C' and C'4 titer supports previous work (4, 6).

Experiments by Dunkerly (2), Ingebrigsten (7), and Schlagenhauf (9) attempted to relate the noted increase in the C'4 titer of guinea pigs and rabbits to the protection which the serum afforded when injected into leukemia infected mice. Ingebrigsten (7) noted that the active role of the specific antibodies present in immune serum were negligible so far as protection to the infected mice was concerned. Dunkerly (2) surmised that the small amount of protection given to the infected mice by rabbit immune serum was accountable by the fact that rabbits normally have an extremely low titer of complement (C') and the component C'4. This was also true of a more closely related species of animals, the Swiss-Webster mice. The relation of C'4 to the immune response was further strengthened by the work of

Renshaw (8), who showed a decreased C'4 titer when cortisone was injected with an antigen. Elliot (3) reported no protection in infected mice by immune guinea pig serum which had also received cortisone at the time of immunization.

The results of the single injection of antiserum (Table IV) apparently do not correlate with the work of previous investigators (2, 7, 9). Perhaps an explanation for these results might be found in the guinea pigs used for this test. Two of the four guinea pigs used did not show an increase in the C'4 titer following immunization. These two animals had previously had injections of cortisone, which has been shown to be inhibitory to any protective capacity of the immune serum.

The results of the repeated serum injections (Tables V, VI, VII, VIII) appear to confirm earlier reports regarding the inability of the specific antibody of the immune serum to increase the survival average of the infected mice. In all experiments using the repeated injections, the serum which had been adsorbed with the antigen prior to injection resulted in an increased survival time when compared to the controls and normal immune serum treated animals. It is significant to note, also, that the presence of antibodies in the immune serum seemed to inhibit the protection of the serum. The animals receiving the unadsorbed serum consistently had a shorter survival time than the adsorbed serum or the controls.

The results of the attempt to show regression of a palpable tumor are shown in Table IX. There was no noticeable regression of the tumor. However, the mice receiving the injections of adsorbed immune serum again showed an increased survival time when compared to the controls and the unadsorbed immune serum. This strengthens the indications of the lack of protection afforded by specific antibodies in the serum and points more to some other factor in the blood.

Table X shows the results of the in vitro tumor inactivation test. This test was significant in the respect that practically all of the mice receiving a mixture of the tumor plus either adsorbed or unadsorbed immune serum failed to develop leukemia. The fact that the adsorbed immune serum was able to inactivate the viable tumor further supports earlier reports that specific antibody is not the protective factor in the immune serum.

One possible explanation of the mechanism involved in the increased protective capacity of adsorbed serum may be found in the ability of complement to react with most antigen-antibody complexes. In adsorption of the immune serum with the tumor suspension in vitro it can be assumed that a part of the total complement enters in the raction. According to Rice and Crowson (1), C'4 in the guinea pig is the highest titered component. The component showing

the lowest titer (C'3 in guinea pigs) regulates the total amount of complement which reacts with the complex. It seems reasonable to assume that the component of highest titer, C'4, will remain in largest amount after the reaction. This might allow the C'4 to be instrumental in the *in vivo* protection which has been described.

This explanation is more feasible in the light of a recent report by Winn (10), who was able to show that the *in vitro* neutralization of 6C3HED tumor cells by mouse isoantiserum was increased by the addition of guinea pig complement. This increased action of the mouse antiserum was not diminished by addition of complement which had been heated to 56 degrees Centigrade. Winn was also able to show *in vivo* inactivation of the tumor cells by the injection of either the heated or normal guinea pig complement. He surmised that the guinea pig complement might act synergistically with some substance of the host. More specifically, he suggested that the guinea pig serum provides some complement components which react with an antibody present in the mouse.

At present, the evidence does not constitute proof that this is the mechanism. However, this is suggested. It is evident that more extensive study is necessary before final conclusions can be reached.

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CHAPTER V

SUMMARY

The results included in this work indicate a relation of increased C'4 titer resulting from specific immunization, to the protective capacity of the immune serum. This protection is not demonstrable in normal immune serum but is shown by adsorbed immune serum. The number of injections of serum apparently does not appreciably alter this protective capacity by showing either an increase or decrease in the survival average, by the techniques used in this work.

Delayed injections of antiserum did not show regression of a palpable tumor. They did show, however, that the adsorbed antiserum was effective in protection after the mice had been infected for a period of seven days prior to the first antiserum injection.

<u>In vitro</u> inactivation of the viable tumor suspension was practically 100 per cent successful. This was significant in showing that the adsorbed immune serum had the same inactivating capacity found in the unadsorbed immune serum. This tends to minimize antibody action in the inactivation of the tumor and to indicate a more complex mechanism responsible for these results.

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