EXPERIMENTAL HYPERSENSITIVITY IN THE RABBIT

Effect of Inhibition of Antibody Formation by X-Radiation and Nitrogen Mustards on the Histologic and Serologic Sequences, and on the Behavior of Serum Complement, Following Single Large Injections of Foreign Proteins[*][‡]

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Recently, the histologic and serologic responses in the rabbit to single, large intravenous injections of purified bovine serum proteins have been described (1). These responses were: (1) *To bovine serum gamma globulin*—The development in most animals of a transient intracapillary glomerulonephritis, together with focal lesions of the liver, heart, and joints, the acute phase occurring from 1 to 2 weeks following injection; (2) *to crystallized bovine serum albumin*— The development in only about half of the rabbits of an intimal and subintimal arteritis of the large pulmonary and systemic arteries, the acute lesions being seen 2 to 3 weeks after injection. When the antigen was gamma globulin, it regularly disappeared from the circulation within 8 to 14 days following injection, and homologous antibody appeared shortly thereafter. In contrast, the albumin disappeared from the circulation after 14 to 21 days and then only in some of the rabbits. In others, it was still detectable after 4 weeks.

From this correlation between the times of disappearance of the antigens, of the emergence of their antibodies, and of the development of lesions, it was deduced that the latter were dependent upon a reaction between antigen and antibody. Furthermore, it was postulated that the difference in distribution of the lesions depended upon a difference in localization of the two proteins used as antigens, since their molecules differ in physical and chemical as well

as antigenic properties. It was further assumed that if the localization of the antigen is the determining factor in the distribution of lesions, the reaction producing the lesions must involve antigen fixed in or on tissue cells and antibody elaborated locally or transported, possibly by cells, from other sites of antibody formation.

The corollary to the conception that these lesions result from the reaction of antibody and fixed antigen is that circulating antigen is of secondary importance to fixed antigen in the pathogenesis of localized tissue damage. This has been clearly demonstrated by Kay (2), who showed that in rabbits, in which antibody formation had been blocked by x-radiation, nephrotoxic serum, prepared by immunization of ducks with rabbit kidney, did not produce renal lesions unless rabbit anti-duck serum was injected subsequently on the 5th, 6th, and 7th days. If the antiserum was injected on the 3 days immediately following injection of the nephrotoxic serum, no renal lesions developed. In both cases, sudden changes in antigen and antibody titres in the serum indicative of an antigen-antibody reaction occurred immediately after injection of the anti-duck serum; but only in the first instance where, presumably, sufficient time had elapsed to permit fixation of the nephrotoxic duck serum in the kidneys, did this antigen-antibody reaction result in renal lesions.

The present study has been undertaken in an effort to determine whether the lesions observed in the rabbit after a single large intravenous injection of foreign protein are, in actual fact, dependent on the formation of antibodies. Two methods—x-radiation and the injection of nitrogen mustards—have been used to suppress antibody formation in the experimental animals, and the tissue responses of these animals after injection with bovine serum gamma globulin have been compared to those of control animals.

In these experiments, bovine serum gamma globulin has been used chiefly as antigen for several reasons. First, it is fairly homogeneous from the immunological as well as the chemical standpoint, unlike whole serum, which has been used in most other studies of this sort. Second, it evokes a uniform serologic response in rabbits. In all animals which we have tested thus far, the antigen has disappeared from the blood within 2 weeks after injection and precipitins have been detectable in the serum very shortly thereafter. Third, it has given rise to tissue lesions with considerable uniformity between 7 and 11 days after injection.

As part of these experiments a study was made of the behavior of serum complement in rabbits after the injection of foreign protein, because of the depression of hemolytic complement activity known to occur in anaphylaxis in guinea pigs (3) and often in human serum sickness (4).

The experimental observations are reported in three sections: (1) Inhibition of antibody production by x-radiation and nitrogen mustards. (2) The relationship between serum complement and serologic sequences; and (3) the relation-

ship between serum complement, serologic sequences, and the development of renal lesions.

The earliest technique for inhibition of antibody formation involved saturation of the reticulo-endothelial system with particulate agents. The literature on this point has been summarized by Jaffe (5). In general the results of this technique have been unsatisfactory. Removal of various organs, particularly the spleen, may diminish, but does not abolish antibody formation.

In 1914, benzol intoxication was found to depress antibody formation (6), and this technique has been further studied by Simonds and Jones (7) and by Hektoen (8).

Since 1908 (9) Roentgen rays have been used to depress antibody production. Literature on this technique has been well summarized recently by Craddock and Lawrence (10). Kay has demonstrated that nephrotoxic nephritis could be prevented in rabbits by a preliminary exposure to x-radiation (2, 11).

Hektoen and Corper (12) in 1921 used mustard gas to inhibit antibody formation in the rabbit. Following the description of the nitrogen mustards in 1946 by Gilman and Philips (13) these agents also were found to depress antibody production in the goat (14) and rabbit (15), and have been used successfully in a case of disseminated lupus erythematosus (16).[1]

In 1942, Cannon (17) advanced the general concept that normal production of antibody (gamma globulin) depends on an adequate intake of essential amino acids. In subsequent experimental work he demonstrated diminished antibody production in rats fed on protein-deficient diets (18, 19). Stoerk and Eisen (20) have shown that pyridoxine is essential for maintenance of normal lymphoid tissue and that prolonged pyridoxine deficiency depresses antibody production in the rat.

In general, the agents having a toxic action on the lymphoid tissue and bone marrow show a marked inhibitory effect on antibody production when used prior to the injection of antigen, but only a negligible inhibitory effect when used after the injection of antigen. Seemingly, the lymphoid-marrow system is essential to initiate antibody formation, but as Craddock and Lawrence have concluded, other reservoirs of antibody must exist.

In the present study, x-ray radiation was selected from the above list of antibody inhibitors because of its convenience; the nitrogen mustards were used largely because of current interest in these compounds.

Material and Methods

Experimental Animals.—Male albino rabbits weighing approximately 2 kilos were used in all experiments.

Whole bovine serum was prepared by Armour and Company.[2] It was sterilized by Seitz

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[1] Since preparation of this manuscript, our attention has been called to the work of Bukantz *et al.* (68) who were able to inhibit the formation of antibody, the development of vascular lesions, and the Arthus phenomenon by the administration of nitrogen mustard to rabbits given a single large intravenous injection of horse serum.

[2] Supplied by the Armour Laboratories, Armour and Company, Chicago, through the courtesy of Dr. Jules Porsche and Dr. James B. Lesh.

filtration and bottled without added preservative. In the preliminary x-ray experiments it was injected intravenously in doses of 10 cc. per kilo. of body weight.

Crystallized bovine serum albumin (lot 17W) was prepared according to the methods of Cohn and Hughes (21) in the laboratories of Armour and Company.[2] It was used in a 25 per cent solution in water to which was added sodium bicarbonate (0.375 Gm. per 100 cc. of solution) giving a final pH of 6.8. The solution was sterilized by Seitz filtration and no preservative was added. This preparation was electrophoretically pure and contained less than 0.05 per cent of globulins (dry weight) by serologic test.

Bovine serum gamma globulin (lots 1823-A3 and C-188 A) was also prepared according to the methods of Cohn and associates (22) by the Armour Laboratories.[2] It was used as a 16.5 per cent solution in 0.3 molar glycine sterilized by Seitz filtration and the acidity was adjusted with a small amount of sodium bicarbonate to give a final pH of 7.0. No preservative was added. These preparations contained 99 per cent gamma globulin by electrophoretic analysis.

Nitrogen Mustards.—Methyl-bis(β-chloroethyl)amine hydrochloride[3] powder was dissolved in physiologic saline to a concentration of 0.5 mg. per cc. This was given intravenously, care being taken to make the injection as soon as possible after the material had been dissolved. The tris(β-chloroethyl)amine hydrochloride was handled in a similar manner. In each experiment the nitrogen mustards were used in amounts sufficient to maintain the total leukocyte counts between 1,000 and 5,000 cells per c.mm.

X-Radiation.—In Experiment 1 the rabbits received x-radiation generated at 200 kv. peak and 18 ma., with filters of 0.5 mm. copper and 1.0 mm. aluminum. The skin target distance was 50 cm. In Experiment 5 the corresponding factors were 200 kv. and 10 ma., through 0.5 mm. copper and 1.0 mm. aluminum filters at a skin target distance of 70 cm., which included approximately 80 per cent of the body surface in the field. In the latter experiment a total of 500 r was given to each animal in a single exposure.

Sheep Cells.—The sheep blood was drawn aseptically in a closed system and preserved in a modified Alsever's solution (23) in a series of small flasks. This procedure was necessary since frequent withdrawals from a large stock solution apparently led to contamination and rapid increase in cell fragility. When used, the cells were washed three times with cold, phosphate-buffered saline. The centrifugation was at 2000 R.P.M. for 5 minutes. The final suspension was approximately 2 per cent, and was standardized by adjusting the concentration so that 1.0 cc. of the final suspension when hemolyzed with 4.0 cc. of distilled water gave a reading of 325 on a Klett-Summerson colorimeter, using the No. 54 filter. This corresponded to a cell concentration of 500,000 cells per c.mm.

Diluent.—Either physiologic saline solution or 0.85 per cent saline solution adjusted to pH 7.3 with phosphate buffer was used throughout the experiments.

Hemolysin.—This was prepared in rabbits according to the usual procedure. The optimal amount for sensitization of sheep cells was determined according to the method of Kent (24).

Complement.—Blood was collected from the marginal ear vein of the rabbits and the tubes placed in ice water until the serum was removed. The sera were sealed with paraffin or sealed in glass ampoules and frozen at −70°C. until used. A standard, pooled rabbit serum, prepared in this manner and frozen in sealed glass ampoules retained a complement titre which varied only 4 per cent in 8 determinations over a period of 6 weeks. All serum samples from a given rabbit were titrated on the same day to eliminate variables such as room temperature and red cell fragility.

Complement Titration.—A modification of the colorimetric method described by Mayer *et al.* (25) and Kent, Bukantz, and Rein (23) was used. The unknown sera were usually diluted 1:10 and added in amounts ranging from 0.4 cc. to 1.4 cc. to six standardized Klett tubes,

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[3] Supplied through the courtesy of Merck and Company, Rahway.

containing 2.0 cc. of sensitized sheep cells. Each tube was brought to a total volume of 5.0 cc. with saline. For each series of titrations three tubes containing 2.0 cc. of cells and 3.0 cc. of distilled water were included to give the colorimeter value for 100 per cent hemolysis. Similarly, three tubes containing 2.0 cc. of cells and 3.0 cc. of saline were included for the reading of 0 per cent hemolysis.

The titrations were carried out in an ice water bath and then incubated for 45 minutes in a 37°C. water bath with occasional agitation. The tubes were then centrifuged for 10 minutes at 2000 r.p.m. The supernatant was read in a Klett-Summerson colorimeter with the No. 54 filter and the percentage of hemolysis calculated for each tube. From these values, the hemolysis unit was determined. Since in the Von Krogh alternation formula (26) the constant, $1/n$ varies greatly with the activity of the sera as shown by Wadsworth, Maltaner, and Maltaner (27) the calculation was obtained by plotting $\dfrac{Y}{1 - Y}$, in which Y equals the percentage of hemolysis, against x, the amount of serum used, on logarithmic paper and determining the 50 per cent hemolysis unit by inspection, a method described by Mayer *et al.* (28). Whenever possible, points above and below 50 per cent hemolysis were used. If only one value between 10 and 90 per cent hemolysis was obtained, the value of $1/n$ in the Von Krogh formula was taken to be 0.14. This was an average of values obtained using the method of least square as described by Kent *et al.* (23) from 30 other satisfactory titrations. Because rabbit serum is low in complement activity (29), the serum itself may interfere with colorimetric determinations; therefore titrations were seldom carried out below a dilution of 1/5.

In the case of all sera with abnormally low complement titres, controls were included in the complement titrations to demonstrate that the low values observed were not due to an anticomplementary effect. The control procedure consisted of heating the sera in question to 56°C. for 50 minutes and adding 0.75 cc. of 1:16 and 1:32 dilutions of this to 1.0 cc. of a 1:100 dilution of guinea pig complement. These mixtures were kept overnight at 3–4°C. and then added to 2.0 cc. of sensitized cells and brought to a total volume of 5.0 cc. with saline. The final mixtures were incubated at 37°C. for 30 minutes and the per cent hemolysis determined in the usual way.

An identical control procedure was run, using serum of normal complement titre from the same rabbit. The per cent hemolysis obtained with the serum of low complement titre was compared with that obtained with control serum of normal titre. If the serum of low titre was anticomplementary, it should reduce the per cent hemolysis below that obtained with the serum of normal complement titre. If the reduction in the value for y was greater than 0.1, the serum in question was considered to be anticomplementary.

Of eleven sera studied in this manner, only one was shown to be markedly anticomplementary and two others were slightly anticomplementary.

Additional controls were run on a representative group of sera of both low and normal complement titre. These consisted of: (1) Heat inactivation of the sera (56°C. for 30 minutes) and subsequent titration of these sera with sensitized cells. Absence of hemolysis eliminates the possibility of hemolysis by substances other than complement. (2) Addition of heat-inactivated serum to non-sensitized sheep cells and subsequent titration of active guinea pig complement, using this cell-serum mixture in place of hemolysin-sensitized cells. Normally no hemolysis should result from this procedure, since no hemolysins are present. If hemolysis occurs, it indicates the existence of naturally occurring hemolysins in the rabbit serum. In no instance were non-complement hemolysis or naturally occurring hemolysins demonstrated.

Cholinesterase.—Serum cholinesterase was determined by the manometric procedure described by Ammon (30), using acetylcholine bromide as substrate and determining the CO_2 evolved per unit of time.

Histologic Studies.—The animals surviving the experiments were sacrificed by air em-

bolism. Necropsies were performed immediately and representative blocks of tissue were fixed in 10 per cent formalin in isotonic saline, Zenker's-acetic acid fixative, and in some instances in Bouin's fixative. Subsequently the blocks of tissue were dehydrated, embedded in paraffin, and sectioned for microscopic study. Histological stains used included eosin and methylene blue (Mallory), hematoxylin, and eosin, and, occasionally Masson's trichrome method.

Inhibition of Antibody Formation

Experiment 1.—

Several series of rabbits were subjected to x-radiation prior to and following the intra-venous injection of whole bovine serum (10 cc. per kilo of body weight) or bovine serum gamma globulin (1 gm. per kilo of body weight). The radiation dosage varied from 150 to 400 r for each single radiation and the number of radiations varied from 5 to 8 given at intervals of several days. Qualitative tests for the presence of antigen and antibody were made 4 weeks following injection in the case of whole serum and 9 days and 2 weeks following injection in the case of gamma globulin. The results were compared with control animals and the serologic findings following similar doses of the same antigens in previous work by Hawn and Janeway (1). The data are summarized in Table I.

It is apparent that, in conformity with earlier findings (9, 10), antibody formation was inhibited by the x-ray dosages employed. Furthermore, there was a definite correlation between the persistence of antigens in the circulation and the failure of antibodies to appear in the serum. Again this is in agreement with similar observations in previous work (9). Not demonstrated in Table I is the fact that approximately 50 per cent of the animals died, apparently from the effects of irradiation. For this reason, in the following experiment nitrogen mustards were used to inhibit antibody formation.

Experiment 2.—

Five rabbits (group A) received the usual intravenous dose of bovine gamma globulin (1 gm. per kilo of body weight). In addition, 3 days prior to injection and the day following injection they received tris(β-chloroethyl)amine hydrochloride in doses of 1 mg. per kilo and a third dose of 0.5 mg. per kilo on the 7th day following injection.

A group of four rabbits (group B) received similar doses of nitrogen mustard without gamma globulin, and a third group of four rabbits (group C) received only gamma globulin, in the same amounts as in the first group. Antigen and antibody titres were determined 1 week after injection and at the time of autopsy, 2 weeks following injection. The results are summarized in Table II.

Four out of five animals receiving both nitrogen mustard and gamma globulin showed antigen in the serum and had failed to develop antibodies by the 14th day. In contrast, all four rabbits in the control group, which received only gamma globulin, developed antibodies by the 14th day and none showed any traces of antigen at that time. In the latter group the characteristic renal lesions were found in three of the four animals, while in the group A animals, receiving nitrogen mustard as well as gamma globulin, no lesions were seen,

TABLE I

Effect of X-Ray Radiation on Antibody Formation

Group	Antigen	No. of animals	Circulating antigen at		Circulating antibody at		Proportion developing antibodies	Proportion with antigen persisting
			2 wks.	4 wks.	2 wks.	4 wks.		
							per cent	*per cent*
X-ray 150–200 r 8 doses	Bovine serum gamma globulin	11	11		0		0	100
Control*	" "	14	0		14		100	0
X-ray 300–400 r 5 doses	Whole bovine serum	7		6		1	14	86
Control*	" "	8		1		7	88	12
			9 days		9 days			
X-ray 150 r 7 doses	Bovine serum gamma globulin	4	4		0		0	100
Control	" "	7	—		6		86	—

* Data from Hawn and Janeway (1).

TABLE II

Effect of Nitrogen Mustard on Antibody Production and Development of Tissue Lesions Following Gamma Globulin Injection

Group	Treatment	No. of animals	Antigen titre		Antibody titre		Proportion with antibodies	Proportion with lesions
			1 wk.	2 wks.	1 wk.	2 wks.		
							per cent	*per cent*
A	Bovine serum gamma globulin	4	4+	3+	0	0		
	+						20	20
	Tris (chlorethyl) amine hydrochloride	1	4+	0	0	3+		
B	Tris (chlorethyl)amine hydrochloride controls	4	—	—	—	—	—	0
C	Bovine serum gamma globulin controls	4	4+	0	0	3+–4+	100	75

except in the one rabbit in which nitrogen mustard had failed to inhibit antibody formation.

With this evidence for the apparent dependence of tissue change on antibody formation at hand, the relationship of serum complement to the serologic changes following heterologous protein injection was investigated.

Relationship between Complement and Serologic Sequences

Experiment 3.—

Four rabbits were injected intravenously with bovine serum gamma globulin (1 gm. per kilo of body weight). Serum complement determinations as well as tests for the presence of antigen and antibody were made every 2 to 3 days for 14 days. Complement activity was determined at similar intervals in a control group of four rabbits who received no injection. Following a suitable recovery period of several weeks, all eight rabbits received an intravenous injection of bovine serum albumin (1 gm. per kilo of body weight) and, similar titrations were made at 2 to 4 day intervals for 22 days. The results for the series receiving gamma globulin are illustrated graphically in Fig. 1.

The control group showed moderate fluctuations in complement activity, but no sudden decreases occurred at any time during the 14 day period of study. In contrast, the four rabbits receiving bovine serum gamma globulin all showed a marked fall in complement activity beginning on the 7th day and reaching its lowest level on the 9th day. In each instance this period of diminished complement activity was correlated with the 2 to 3 day period during which antigen was disappearing from and antibody was appearing in the circulation.

The results following the injection of crystallized bovine serum albumin are charted in Fig. 2. It will be seen that in three animals antibodies had failed to develop by the 22nd day, and antigen was still present in the circulation at that time. In these three animals complement levels remained essentially unchanged during the course of the experiment; while in the five animals which developed antibodies a sudden fall in complement titre occurred. The time of this fall ranged from the 9th to the 20th day and in every instance antigen persisted in the circulation during the period when complement activity began to diminish, while antibodies became demonstrable in the serum at about the time the complement titres had returned to their previous levels.

Thus, a correlation between sudden drops in serum complement titre and presumptive evidence of interaction between antibody and antigen has been established. If this correlation is valid and if the fall in complement depends on an antigen-antibody reaction, it would follow that by preventing the reaction between antigen and antibody by inhibiting antibody formation, the fall in serum complement should be prevented. The following experiments were undertaken to investigate that question, as well as to demonstrate a possible correlation between the changes in complement titre and the development of tissue lesions.
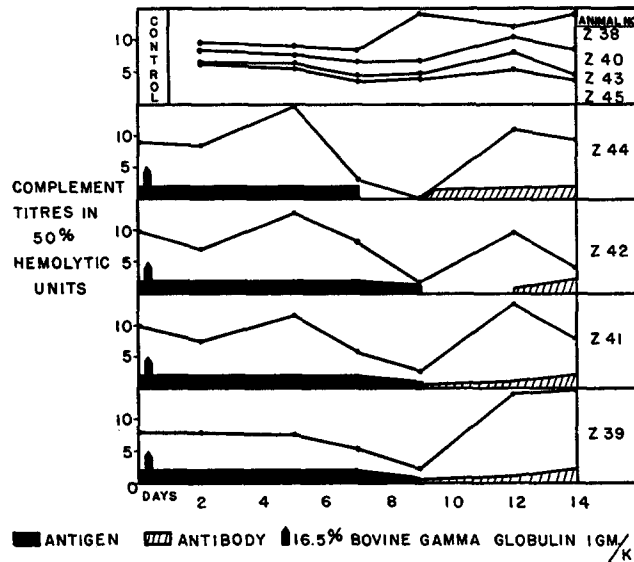
FIG. 1. Serum complement titres following intravenous bovine serum gamma globulin. Note that lowest complement titre occurs on 9th day after injection of bovine gamma globulin in each instance and that titre rises as antigen disappears and antibody appears.
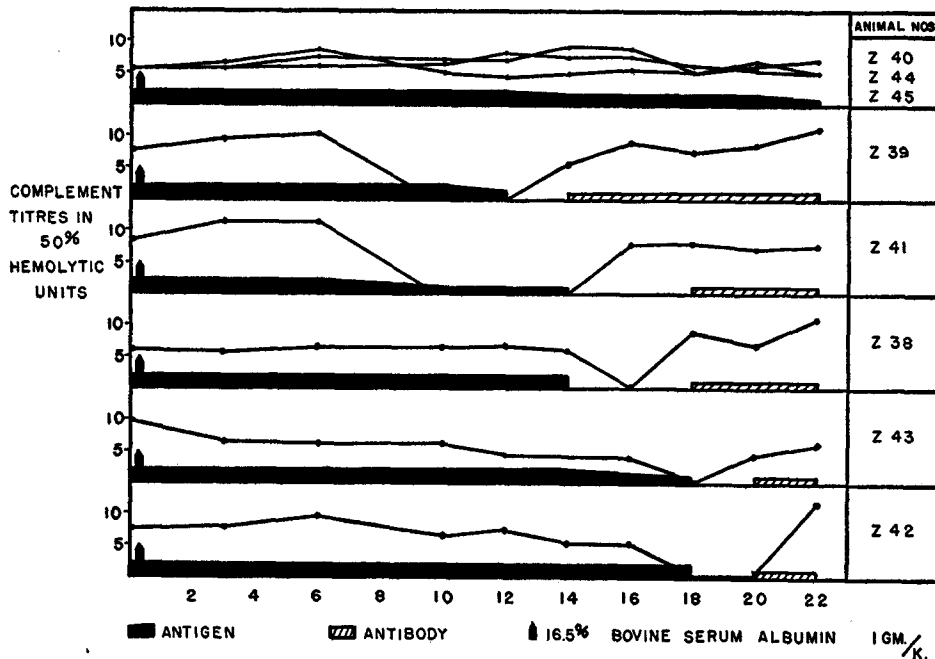

FIG. 2. Serum complement titres following intravenous bovine serum albumin. Bovine serum albumin was given after several weeks' rest to the same group of rabbits shown in Fig. 1. Note that in three rabbits antigen remained in circulation for 22 days without fall in serum complement. Note variable and longer interval between time of injection and fall in serum complement, but note that lowest titre bears the same relation to time of disappearance of antigen and appearance of antibody as in Fig. 1.

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Relationship between Complement, Serologic Sequence, and Histologic Changes

Experiment 4.—

A group of nine rabbits (group A) was given a single intravenous injection of bovine serum gamma globulin (1 gm. per kilo of body weight) and serum complement, antigen, and antibody titres were determined at 2 to 4 day intervals.

A second group of four rabbits (group B) was given a similar intravenous injection of bovine serum gamma globulin and, in addition, nitrogen mustard (methyl-bis(β-chloroethyl) amine hydrochloride) was given according to the dosage schedule I detailed in Table III. Serum complement activity and the presence or absence of antigen and antibody were determined at 2 to 4 day intervals.

A third group of six rabbits (group C) was treated similarly, except that larger amounts of nitrogen mustard were given in the period following the gamma globulin injection (see schedule II of Table III).

TABLE III

Nitrogen Mustard Dosage Schedule

Schedule	Drug used	Days before antigen						Days after antigen									
		Dosage *mg.*															
		6	5	4	3	2	1	0	1	2	3	4	5	6	7	8	9
I	Methyl bis(chloro-ethyl)amine HCl		2.0	2.0	2.0	2.0			0.25					0.5			
II	" "	2.0	2.0	2.0					1.0			0.5		0.5	1.0		
III	Tris(chloroethyl) amine HCl								1.0			1.0				1.0	

A fourth group of three rabbits (group D) was handled similarly except that the nitrogen mustard used was tris(β-chloroethyl)amine hydrochloride, given according to dosage schedule III of Table III.

Finally, a fifth group of seven rabbits (group E) received only nitrogen mustard injections: two according to schedule I, three according to schedule II, and two according to schedule III. All twenty-nine animals were sacrificed on the 9th to 11th day following injection of gamma globulin and histologic studies were made.

The results with reference to the behavior of serum complement and the development of tissue lesions in the five groups of animals studied are summarized in Fig. 3.

It will be seen that in those animals receiving only gamma globulin, complement activity began to decline on the 7th day and reached its lowest level on the 9th and 10th days. Typical glomerular lesions were demonstrated in all animals sacrificed from the 9th to 11th day. In group B, in spite of the administration of nitrogen mustard, a similar fall in complement was observed, and in three of these four animals antibodies were detectable by the 11th day. In contrast to groups C and D, the average leukocyte counts following nitrogen
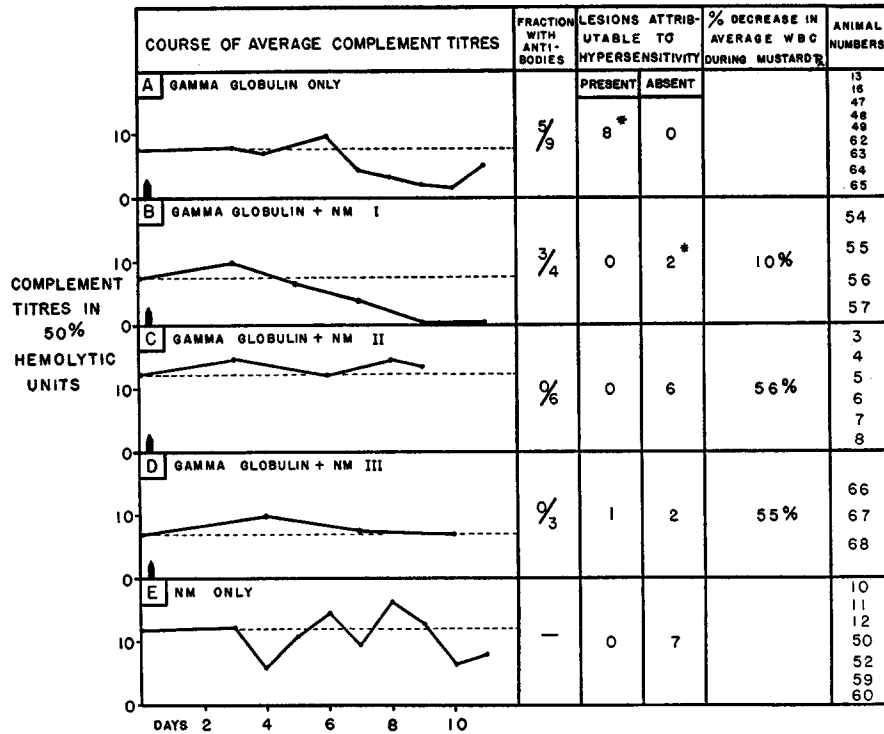
FIG. 3. Effect on complement titres of inhibition of antibody production by nitrogen mustards. Summary of Experiment 4. Note similarity of behavior of serum complement in A and B to behavior shown in Fig. 1. Fall in serum complement in B presumably was due to failure of nitrogen mustard to inhibit antibody formation or to hold down white blood count as in C and D, where complement titre did not fall, there was no antibody formation and white blood count decreased markedly.

mustard treatment were only slightly lower than the average counts previous to treatment. Moreover, the counts rose sharply to supernormal levels at one point. These facts suggest that inadequate dosage was probably the reason for the failure of nitrogen mustard to prevent the formation of antibodies in this group.

The pathologic findings in group B are limited to two animals, since the remaining two died and were not autopsied. The two animals studied both failed to show lesions of any type. In one of these the serum gave a 1+ antibody test and in the other antibodies failed to develop by the 11th day. However, in this animal the complement level fell in the same manner as in rabbits in which circulating antibodies subsequently appeared. Thus, in two animals in which, judging from the serologic reactions, tissue lesions should have been expected to develop, none were found. The several possible interpretations of this discrepancy will be discussed later.

In contrast to these results, the six animals in group C, receiving larger amounts of nitrogen mustard, did not develop antibodies, did not exhibit any fall in complement titre by the 9th day, and failed to develop any tissue changes. The greater effectiveness of this larger dosage of nitrogen mustard can also be seen in the significantly lower average leukocyte count following nitrogen mustard administration. In group D, again the complement titres showed no significant decline and antibodies did not appear by the 10th day. However, in this series, one of the three animals developed histologic evidence of acute glomerular damage.

The control group E, receiving only nitrogen mustard, failed to show any significant changes in complement titre other than rather marked fluctuations around a mean titre of about ten units, and showed no histologic evidence of significant toxicity.

The results of the serologic determinations are summarized in Table IV. Comparison between the animals developing tissue lesions and those showing no tissue changes is difficult, as far as the persistence of antigen and the development of antibodies is concerned, because of the fact apparent in Table IV, that the serologic determinations in the latter group were made on the 9th day, while in the former group they were done on the 11th day. This discrepancy between the two groups undoubtedly accounts in part for the greater frequency with which antigen was present and antibody was absent in the animals failing to develop renal lesions; nevertheless, the differences between the two groups are consistent with the results of Experiment 2 and the data previously presented (1).

In spite of minor discrepancies, this evidence indicates that the nitrogen mustards are capable of suppressing antibody formation, the characteristic decline of complement activity, and the development of tissue lesions. It seems probable that the failure of lesions to develop is due to the absence of anti-

bodies, but a prophylactic effect of the nitrogen mustards independent of suppression of antibody formation cannot be excluded. If, however, similar results could be obtained by the use of completely different antibody-suppressing

TABLE IV

Comparison of Pathologic and Serologic Changes Following Nitrogen Mustard Administration

Group	Treatment	Antigen titre			Proportion with antigen	Antibody titre			Proportion with antibody	Proportion with lesions attributable to hypersensitivity
		9 days	10 days	11 days		9 days	10 days	11 days		
				per cent					*per cent*	*per cent*
A	Gamma globulin			0 1+ 0				1+ 2+ 3+		
B	Gamma globulin + Nitrogen mustard (I)			2+ 2+ 0 2+	69			2+ 0 1+ 1+	62	80
A	Gamma globulin	4+	0 1+ 4+ 4+ 4+			0	2+ 2+ 0 0 0			
D	Gamma globulin + Nitrogen mustard (III)		4+ 4+ 2+				0 0 0			
	Gamma globulin + Nitrogen mustard (II)	4+ 4+ 4+ 4+ 4+ 4+			100	0 0 0 0 0 0			0	11

agents, it could then be argued more logically that the absence of antibodies and not the antibody-suppressing agent *per se* is the cause of the prophylactic effect observed on the development of lesions.

For this reason, an experiment similar to Experiment 4 was attempted, using x-radiation to inhibit antibody formation. A single large exposure was adopted to decrease the mortality encountered in Experiment 1.

Experiment 5.—

A group of twelve rabbits was treated as follows:—Four animals were given single injections of bovine serum gamma globulin (1.0 gm. per kilo of body weight in 16.5 per cent solu-
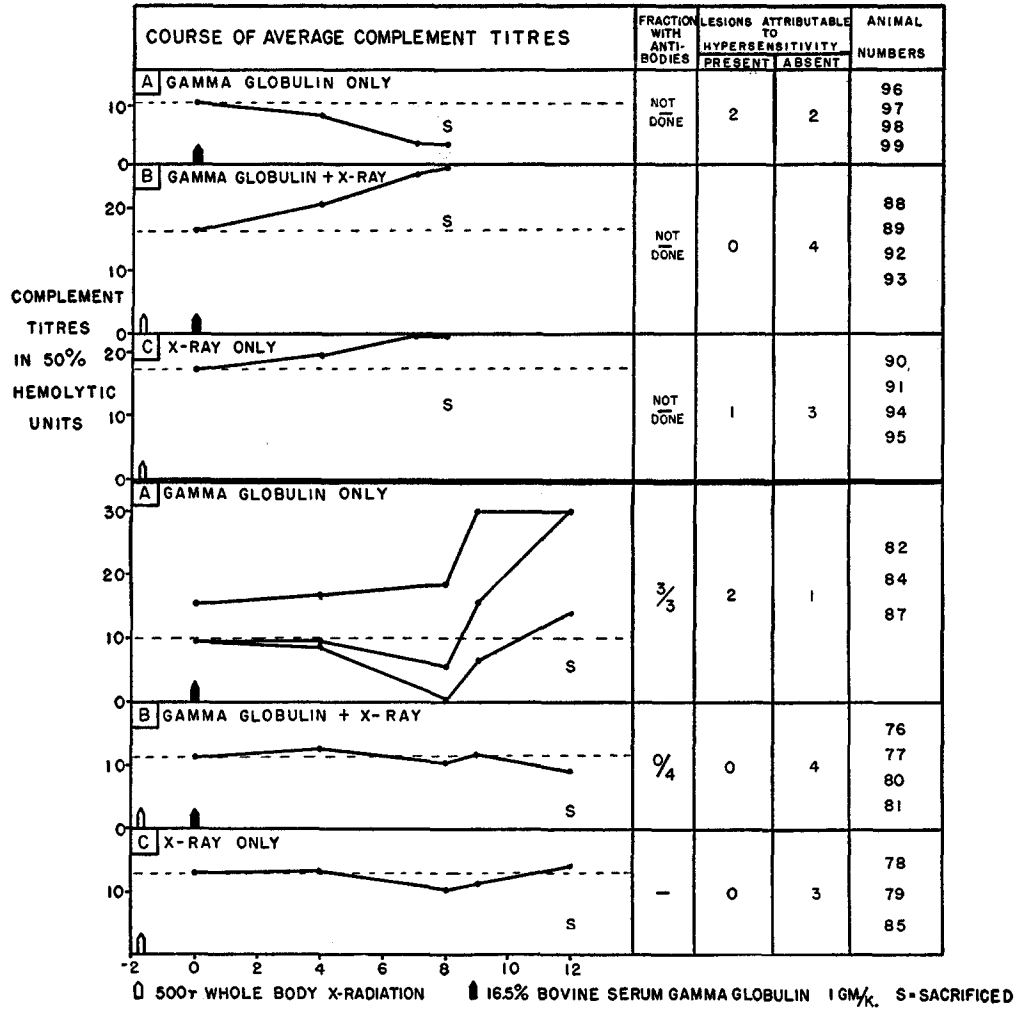

Fig. 4. Effect on complement titres of inhibition of antibody production by x-radiation. Summary of Experiment 5. Note that when x-radiation prevented antibody formation or the development of definite lesions, serum complement titre did not fall.

tion); four others received 500 r whole body radiation 2 days prior to the injection of the same amount of bovine serum gamma globulin; and, finally, four other animals were irradiated with the same Roentgen dosage, but received no protein injections. Complement activity was determined immediately before injection and periodically thereafter. All animals

were sacrificed for histologic study on the 8th day, since lesions are apt to be most marked at this time, even though antibodies may not be detected consistently until the 10th or 11th day.

A second group of ten rabbits was handled in the same manner, except that they were not sacrificed until the 12th day, to permit more significant tests for the presence or absence of antibodies. The results are summarized in Fig. 4.

Again, the pattern seen in Experiment 4 is clear. The x-radiation has inhibited antibody formation in all four cases where tests were made. It has prevented a fall of complement in eight rabbits, in contrast to its occurrence in six of seven controls. It has prevented the development of significant renal lesions in all eight animals studied; as compared to the occurrence of such lesions in four of seven controls.

In conjunction with Experiment 4 these results point to the suppression of antibodies as the cause of the failure of tissue lesions to develop.

DISCUSSION

Since it was the intent of our previous report (1) to describe in detail the histologic sequences resulting from the injection of purified protein antigens and to correlate these with simultaneous serologic studies, complete histologic description has not been attempted in the present report. Furthermore, the histologic changes induced by x-radiation and certain of the nitrogen mustards have been fully described and will not be considered further here. In our experience, the glomeruli of animals subjected to x-radiation or the injection of nitrogen mustard alone may show slight increase in intrinsic cellularity and a slight increase in intracapillary leucocytes over the number encountered in untreated control animals.

The histologic interpretations in the present experiments were reached without prior knowledge of the serologic findings. In the animals receiving antigen alone, changes attributable to hypersensitivity were seen in kidneys, lungs, and heart. Synovial tissues were not studied. Glomerular lesions were the most constant finding, and the most readily interpreted, while myocardial and endocardial lesions were present in only a small percentage of cases. Cardiac changes were never present in the absence of renal lesions. In the tables, the notation of the presence of presumptive histologic evidence of hypersensitivity indicates that unmistakable renal changes were seen.

Lesions within the pulmonary vessels such as have been described by Ehrich, Seifter, and Forman (31) were difficult to interpret. Perhaps the observed nonspecific endothelial swelling, proliferation, and leukocyte infiltration are the results of a local toxic effect of the initial high concentration of intravenously injected protein or nitrogen mustard. In those animals showing renal and cardiac lesions, these pulmonary changes usually were more severe. It seems possible that tissue changes of hypersensitivity may have been superimposed on preexisting foci of non-specific tissue damage.

It must be stressed that the demonstration of convincing histologic evidence of glomerular alteration depends on the time interval chosen for sacrifice of the animal, since these changes are apparently reversible or reparable (1). The absence of lesions in animals sacrificed after the 14th day cannot be interpreted since healing might have been expected by that time. The failure of Dammin and Bukantz (32) to demonstrate glomerular lesions may have been due to the fact that their rabbits were not sacrificed until the 17th day after injection of bovine serum gamma globulin. More and Waugh's (33, 34) modification of the technique used here to produce renal lesions may minimize these difficulties.

Evidence has been presented that x-radiation and nitrogen mustards inhibit antibody formation and when used prior to intravenous injections of bovine serum gamma globulin, the tissue lesions ordinarily seen 7 to 10 days thereafter generally fail to develop. These facts support the thesis that these lesions are directly related to the development of antibodies and are not merely toxic reactions to foreign protein. However, the results of Experiment 4 leave this conclusion open to question. In the group B animals, which received inadequate amounts of nitrogen mustard, lesions failed to develop in two cases, in spite of the formation of antibodies. This suggests that the nitrogen mustards may prevent the development of lesions by some means other than inhibition of antibody formation. If that were true, lesions probably should have been prevented in all animals receiving comparable amounts of nitrogen mustard, regardless of the serologic changes. That that was not the case can be seen from Table II.

It has also been demonstrated that there are close correlations in time between depression of serum complement and disappearance of antigen from the circulation on the one hand, and on the other, return of serum complement to normal levels and the appearance of homologous antibody, and further that there is a correlation between these events and the development of tissue lesions. Before discussing the significance of these findings, it is necessary to mention the factors which must be considered in interpreting any depression of serum complement titre.

Seitter and Ecker (35) have shown that during proteinuria in man complement is excreted in the urine. The absence of proteinuria was demonstrated in eight of our animals at the time of autopsy, when complement titres were abnormally low. Furthermore, Thomson et al. (36) and Kellett (37) found that in acute and chronic human glomerulonephritis the degree of albuminuria bore no relationship to complement titres.

The possibility of an anticomplementary effect also must be considered. However, the anticomplementary control tests (see Materials and Methods) have eliminated this possibility in the great majority of cases. The anticomplementary effect of gamma globulin described by Davis et al. (38) and by Thomas

and Dingle (39) should have been observed immediately following the injection of the bovine gamma globulin—not 9 days later.

It is well established that during liver disease in man, low complement titres occur. This is presumably due to an anticomplementary effect which can be demonstrated and correlated with the occurrence of abnormal globulin (40). The level of serum cholinesterase is a rather delicate test of hepatic function, low values being obtained in patients with hepatitis or cirrhosis of the liver. Determinations of serum cholinesterase on pooled sera from Experiment 4 showed no significant differences between the animals developing lesions following gamma globulin injection, those in which lesions were prevented by nitrogen mustard administration, and those receiving only nitrogen mustard. Those results can be taken as a rough indication that liver function was not disturbed seriously during the course of the disease process in our animals.

Complement activity has been shown to be a function, within certain limits, of magnesium ion concentration (25). If the transient decrease in complement titres observed here were due to a sudden decrease in serum magnesium concentration, the fall in complement titre could not be demonstrated if complement titrations were carried out in the presence of an added excess of magnesium. This procedure was used in retitrating serial samples of sera from four rabbits in which the characteristic sudden fall in complement titre had been demonstrated. Each point on the curves obtained in the presence of excess magnesium was at a level approximately 75 per cent higher than the corresponding points on the curves obtained from titration without added magnesium. However, the sequential changes in complement titre were the same in both series of titrations.

The assumption, then, can be made, through exclusion, with a fair degree of certainty that the fall in complement titre observed in our animals is due to fixation of complement by an *in vivo* reaction between antigen and antibody.

The most recent work suggesting the occurrence of *in vivo* complement fixation is that of Stavitsky, Stavitsky, and Ecker (41). Transient hypocomplementemia was observed consistently following reinjection of a variety of antigens in specifically sensitized rabbits. Definite correlation was demonstrated between this phenomenon and the presence of circulating antibodies. During the course of acute anaphylactic shock in guinea pigs, serum complement titres are depressed to varying degrees (3, 42, 43). However, Johns (44) has pointed out that frequent bleedings and non-specific foreign serum injections also depress complement activity in the guinea pig. In the rabbit, Friedberger and Hartoch (3) failed to demonstrate a fall in complement titre during anaphylaxis; however, such a relationship has been observed in this laboratory.

In human serum sickness low complement titres were found by Rutstein and Walker (4) during the acute phase in eight of sixteen cases. Gunn (45) found that a fall in complement titre coincided with the probable formation of antibodies in enteric fevers; and Ogawa and Sato (46) demonstrated a fall in complement titre at the

onset of nephrotoxic nephritis in the rabbit, a condition which Kay (2, 11) has demonstrated to be dependent on an antigen-antibody reaction.

If the depression of complement seen in the present experiments is due to a specific fixation and can be taken as an index of antigen-antibody reaction, the peak of that reaction occurs at approximately the 9th day. This would further support the concept that the acute lesions, seen at approximately the same time, are the result of antigen-antibody interaction.

It is of interest to compare the findings in the present controlled experiments with the behavior of serum complement in certain clinical syndromes commonly regarded as being due to hypersensivtiity to some as yet unidentified antigen.

Kellett and Thomson (47) have shown that a low complement in glomerulonephritis is a consistent finding only in the acute phase. Thomson *et al.* (36) have confirmed these results, and we have been able to duplicate these observations. Rachmilewitz and Silberstein (48), as well as previous workers (49, 50), found low complements titres during the acute phase of rheumatic fever. However, de Gara and Goldberg (51) could demonstrate low complement titres in only 25 per cent of 29 children with active rheumatic fever, and found low titres in the same proportion of 75 children during acute non-rheumatic illnesses. Nevertheless, they demonstrated diminished complement in a significantly greater proportion of active than of inactive rheumatic fever patients.

Schnabel (52) and Veil (53) found low complement titres in 45 per cent of cases of acute arthritides. Others, notably Paul and Pely (54) found decreased complement titres in acute allergic syndromes such as asthma and urticaria, while Tilden (55) failed to find such changes in chronic asthma and chronic hay fever.

This combined evidence has led to a general assumption that in the absence of hepatic failure or infection, where complement values may also be greatly depressed (56), a diminished serum complement titre is presumptive evidence of an *in vivo* antigen-antibody reaction as the possible basis for the disease in question.

The present correlation of a fall in complement with serologic evidence of an *in vivo* antigen-antibody reaction provides supporting evidence for such a concept, and emphasizes the need for further study of the role complement may play in the cellular damage associated with anaphylactic states.

The evidence presented here can also be considered as bearing on the etiology of nephrotoxic nephritis. Ogawa and Sato (46) have found a diminished complement, coincident with urinary signs of acute nephritis, 7 to 9 days following the injection into rabbits of nephrotoxic duck serum. Kay (2, 11) has shown that nephrotoxic nephritis depends on active production of antibodies for duck serum, rather than on passive transfer of antikidney antibodies. The behavior of complement in the present experiments is identical to that observed by Ogawa and Sato and substantiates the conclusions to be drawn from Kay's work that there is no fundamental difference between nephrotoxic nephritis and the serum-sickness type of nephritis produced in the present experiments.

In the introduction, reasons were given for considering the focal character of the observed lesions as due to some chemical property of the antigen which leads to its fixation in certain specific sites. A possible hypothesis is that the chemical nature of the antigen results in the formation of a complex with tissue protein which in turn incites the formation of antibodies capable of reacting with the normal tissue protein. Such "autoantibodies" have been demonstrated by Cavelti (57) in rats with homologous kidney extracts incubated with streptococci and by Lange et al. (58) in human glomerulonephritis. We have not looked for them in our animals, but More and Waugh (33) were not able to find renal autoantibodies in rabbits, in which they produced glomerulonephritis by injections of bovine serum globulin. However, for this purpose they used the ring test previously described (1), not the more sensitive collodion particle technique employed by Lange et al. (58).

It is pertinent to speculate, on purely theoretical grounds, on the role complement may play in the pathogenesis of the tissue lesions resulting from the injection of foreign protein. These lesions are thought to depend on an interaction between antibody molecules and whole cells, rendered peculiarly reactive to that antibody by reason of antigen fixed on or within them. This reaction is analogous to that between normal sheep cells and specific anti-sheep cell hemolysin. In the absence of serum complement, the latter reaction produces no evident damage to the sheep cells, but in the presence of complement gross cellular destruction occurs. By applying this analogy to the present experiments, it is possible to consider the reaction between antibody and the antigen-tissue cell complex as merely sensitizing the cells to the subsequent destructive action of serum complement. This concept would give to complement an active role in the production of cellular damage during an *in vivo* antigen-antibody reaction. This is not to imply that the decrease in complement activity observed here can be accounted for by the small quantity of complement which might be fixed by tissue cells during such a process. The reaction between circulating antigen and antibody is probably quantitatively much more important.

While admittedly theoretical, such a concept is supported by experimental evidence.

Kulka (59, 60) demonstrated that the isolated *normal* guinea pig uterus, passively sensitized by contact of Ringer's solution with antiserum for type 3 pneumococcus polysaccharide, will contract, on the addition of the specific antigen, only in the presence of complement.

Similarly, Witebsky and Neter (61) demonstrated that sheep call antiserum (containing induced Forssman antibodies) lost its lethal effect on chick embryos (carrying Forssman antigen) when inactivated at 56° C., for 30 minutes, and that this lethal action could be restored by the addition of small amounts of fresh guinea pig complement, which in itself had no traumatic effect on the embryo. These results were confirmed by Bier and Seiler (62). Watanabe (63) also has indicated that fresh guinea pig serum may increase the toxicity of specific rabbit antisera for *Paramecia*.

In contrast to this evidence for an active role of complement in anaphylactic reactions are the classic experiments of Dale (64), demonstrating that the isolated uterine horn of the sensitized guinea pig will contract on addition of the sensitizing antigen, apparently in the absence of complement. Similarly, Hyde (65) has been able to produce anaphylactic shock in a single guinea pig from a strain totally deficient in the third component of complement. However, in the case of the Schulz-Dale phenomenon it might be questioned whether complement is not fixed to the tissue cells in the same manner as the specific antibody obviously is, a possibility supported by the theory of Heidelberger and coworkers (66, 67) that complement is loosely bound to antibody molecules prior to the antigen-antibody reaction.

It is clear from this evidence that the question of the part complement plays in anaphylactic phenomena is not a simple one, but the possibility that it may play a vital role in the pathogenesis of tissue lesions in hypersensitive states cannot be overlooked in our efforts to define this process in exact terms.

SUMMARY

X-radiation and nitrogen mustard administration inhibit the formation of precipitins for whole bovine serum and bovine serum gamma globulin in the rabbit.

When specific antibody formation is inhibited by these agents, intravenous injection of a single large dose of bovine serum gamma globulin is not usually followed by the development of tissue lesions 9 to 11 days later, as occurs fairly regularly in control animals.

A fall in titre of serum complement to very low levels for 3 to 5 days is closely correlated in time with the disappearance of antigen from the circulation following the intravenous injection of single large doses of bovine serum albumin and bovine serum gamma globulin. A rise in complement titre to normal levels occurs as antibodies appear in the serum.

This sudden fall in complement titre is correlated with the development of characteristic lesions, and does not occur when antibody formation is inhibited.

The data presented are interpreted as evidence in favor of the concept that the lesions are due to a reaction between antigen fixed in or on tissue cells and circulating antibody.

The possible significance of serum complement in the pathogenesis of anaphylactic tissue lesions is discussed.

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