THE ROLE OF COMPLEMENT IN THE PASSIVE CUTANEOUS REACTION OF MICE

By S. BEN-EFRAIM,* Ph.D., and B. CINADER, D.Sc.

(From the Departments of Medical Biophysics and Pathological Chemistry, University of Toronto, and the Subdivision of Immunochemistry, Division of Biological Research, Ontario Cancer Institute, Toronto, Canada)

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Insight into the consequences of the *in vivo* interaction between antigen and antibody depends on the analysis of systems in which relatively simple chains of reactions can be studied. One such system is passive cutaneous anaphylaxis (PCA), an immediate type of skin reaction (1, 2), which is initiated by a combination between antigen and cell-fixed antibody in the skin and characterized by increased permeability of the walls of minute blood vessels in the skin. The first of the events leading to this increase in permeability is the attachment of antibody to cells in the intradermally injected site of the skin (3–7). The next event is the attachment of antigen to the fixed antibody. After this a series of further reactions culminates in increased permeability of blood vessels. The question arises as to what these further reactions are, and, in particular, whether complement is involved in them. So far, most attempts to elucidate this question were based on experiments with animals in which the circulating level of complement was reduced experimentally. Such reduction can be caused by systemic injection of antigen-antibody precipitates before the injection of the reactants, leading to PCA (8–10). Another approach has been based on chemical modifications of antigen which result in reduced complement fixation by the complex of antigen and antibody. On the basis of such experiments it was concluded that complement is involved in PCA of the rat (8, 9), but not of mice (11).

The role of complement in PCA of the guinea pig was examined by a third type of analysis: it is possible to separate antibody into a fraction which fixes complement and another which does not. Experiments with such preparations are consistent with the view that complement is not involved in PCA of the guinea pig (10).

There are other cutaneous immunological reactions in which a role of complement has been postulated; one of these is the reverse passive Arthus reaction (RPAR), so far studied in the rat (12), rabbit (13, 14), guinea pig (7, 10, 15), and mouse (16). The RPAR has been differentiated from PCA by its histopathology, is caused by aggregates of antigen and antibody, and does not involve the fixation of antibody to cells (17, 18). It is thus characterized by the fact that it can be elicited by antigen, administered simultaneously with the intradermally injected antibody. Unlike PCA, RPAR does not depend on the fixation of antibody on cellular sites. In the context of our present

* Present address: Israel Institute of Biological Research, Ness Ziona, Israel.

paper only the first stage of RPAR can be studied and will be defined as the permeability increase which occurs when antigen and antibody are injected simultaneously.

As has already been indicated, past experiments on the role of complement in the various immune cutaneous reactions had to be carried out with animals temporarily deprived of complement. Such deprivation was brought about by the injection of antigen-antibody complexes, of aggregated gamma globulin (19, 20), or of various chemical reagents (21). The biological effect of such agents is, however, not confined to the complement system but extends to various cellular elements (22). It is, therefore, always questionable whether changes in the reactivity of the experimental animal are attributable to one or all of several biological effects of the pretreatment. It would therefore be of value to investigate the effect of complement with animals which are genetically deficient in complement. Complement-deficient guinea pigs (23) and complement-deficient rabbits (24) have been described, but these animals could not be produced in sufficient numbers to allow extensive experimentation. Recently, it has, however, become evident that whereas some strains of mice possess a complete complement system (25), other strains of inbred mice lack one factor of the complement system (26–28). The discovery of an antibody by which an antigen, MuB1, can be detected has allowed us to demonstrate that the absence of this antigen can be correlated with a hemolytically non-functional complement system (28–31). This has facilitated an extensive survey of inbred strains of mice for the presence or absence of a hemolytically functional complement system, and thus it has been possible to classify inbred strains as complement-positive or complement-negative (30, 31). On the basis of this information the role of complement can be investigated in a variety of pathological conditions caused by antigen-antibody interaction.

The PCA reaction can be demonstrated in mice. Ovary (32) who used egg albumin and egg albumin antibody (rabbit), found that the reaction could be detected with 0.4 to 0.5 μg N antibody and that the optimal interval between the intradermal injection of antibody and the intravenous injection of antigen was 3 hours.

It is the purpose of the following experiments to investigate the role played by complement in passive cutaneous reactions (PCA) of mice which have been shown to possess the antigen MuB1 and of mice which have been shown to lack this antigen.

The reactants chosen for the following study were crystalline bovine ribonuclease and the antibody to this antigen, raised in rabbits. Ribonuclease has been previously shown to be able to elicit PCA (9), and its choice in our experiments was motivated by the extensive information available in our laboratory on the interaction between ribonuclease and the corresponding antibody (33, 34).

Materials and Methods

Materials.—

Mice: Of the 18 strains of inbred mice used, 17 were obtained from Roscoe B. Jackson Memorial Laboratories, Bar Harbor. Strain SL/R1 was obtained from Dr. Liane B. Russell, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee. Non-inbred strains of Swiss mice, all MuB1-positive, were obtained from the Connaught Institute, Toronto. The

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following MuB1-positive mice were employed (29): BALB/cJ, BDP/J, BUB/Bn, CBA/J, C57BL/6J, C57BR/cdJ, C57L/J, C58/J, PL/J, SJL/J, SL/R1, 129/J, Swiss. The following MuB1-negative mice were employed (28): A/HeJ, AKR/J, CE/J, DBA/2DeJ, RF/J, SWR/J.

Guinea pigs: Animals of Hartley strain were obtained from Mr. Fred Geissen, New York.

Normal rabbit sera (NRS): Sera were obtained by separation from the pooled blood of 15 to 20 normal adult rabbits, bled from the carotid artery. Before use, the rabbit sera were kept for 1 hour at 56°C.

Normal guinea pig sera (NGPS): Blood was obtained by cardiac puncture of inbred guinea pigs (Hartley strain). Sera were separated and used on the day on which the blood was obtained. Blood and sera were kept at +2°C from the moment the blood was shed to the time the sera were used.

Ribonuclease antiserum: Rabbits were immunized by subcutaneous injection with bovine pancreatic ribonuclease incorporated in Freund's adjuvant; followed by a course of intravenous injections as described previously (29). The serum used in these experiments was 801 (13/2/63). It contained 1.0 mg of antibody N/ml.

Ribonuclease: Bovine pancreatic ribonuclease was obtained from Sigma Chemical Co., St. Louis (crystallized 5 times) Lot 6 3B-1020. Activity, 43 Kunitz units/mg.

Diluent: Reactants were diluted with 0.15 M sodium chloride.

Dye: Evans blue was obtained from Fisher Scientific Company. The dye (1 per cent w/v) was diluted in 0.15 M sodium chloride.

Methods.–

Quantitative precipitin test: The antibody content of ribonuclease antiserum 801 was determined by quantitative nitrogen assay of antigen-antibody precipitates (29).

Bleeding of mice: Mice were bled from the tail. The animals were placed in plastic tubes which had an air hole punched in the normally closed part of the tube. At the opposite end, the tube was closed with a neoprene stopper. This stopper had a central hole, through which the tail of the mouse was threaded. The tail was then immersed in warm water, dried with filter paper, and the end was snipped off.

A test tube (height, 50 mm; diameter, 9 mm) surrounded by an ice-water bath was placed in a conical flask which was evacuated. Blood was shed into the test tube. The tubes containing blood were kept in an ice-water bath for about 1 hour and were then centrifuged at 0–2°C. The serum was then separated and was centrifuged again until free of erythrocytes. Sera were used immediately after separation.

Intradermal injections: The backs of mice were shaved with an electric hair clipper (Oster model A2-22 small). The total volume injected into any one skin site was 0.025 ml. One injection was administered on each side of the animal with syringes of a capacity of 0.25 ml fitted with needles ⅜ inch in length and 27 gauge in width.

Intravenous injections: Ribonuclease and Evans blue were mixed immediately before the injection (final concentration of Evans blue was 0.5 per cent w/v). 0.25 ml of this mixture containing 100 μg N of ribonuclease was injected into the tail vein of each mouse (needle: 27 gauge, ⅜ inch in length).

Test for presence of MuB1: Sera were tested for the presence or absence of antigen by double diffusion in agar against a rabbit serum or mouse serum specific for MuB1 (28).

Test for passive cutaneous anaphylaxis (PCA): Intradermal injections with ribonuclease antibody were followed 2.5 to 3.0 hours later, if not otherwise stated, by intravenous injection of a mixture of Evans blue and ribonuclease. In some experiments the mixture of ribonuclease and Evans blue was injected first and the intradermal injection of antibody was administered immediately afterwards. In all experiments the antigen quantity administered was 100 μg N ribonuclease. 1 hour after the intravenous injection the mice were sacrificed. The skin was

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removed and the diameter of the blue area on the inner surface of the skin was recorded as the mean of two measurements at right angles.

Test of the effect of intravenously administered serum on the reaction of PCA: Sera from normal animals were mixed with ribonuclease and Evans blue immediately before intravenous injection. Intravenous injections and other technical procedures were carried out as described above.

RESULTS

The intradermal injection of ribonuclease antiserum followed by the intravenous injection of a mixture of ribonuclease and Evans blue led to the ap-

TABLE I

PCA Response in Various Strains of Mice

All animals were males, 6 weeks of age. Intravenous injection: 100 μg N ribonuclease.

Strain	MuB1	Average diameter of response						NRS per skin site
		Ribonuclease antibody injected intradermally, μg N antibody per skin site						
		1	2.5	3	6	12.5	25	
		mm	*mm*	*mm*	*mm*	*mm*	*mm*	
BALB/cJ	+		0.8 (9)	5.8 (5)	8.8 (3)	8 (3)	11.5 (2)	0 (2)
BUB/Bn	+			5.5 (6)	5.8 (6)	8.7 (4)	10.0 (2)	0 (2)
SJL/J	+			8.8 (11)	10.4 (9)	11.2 (9)	12.8 (8)	0 (8)
Swiss	+	0.6 (12)	0.8 (9)	4.9 (10)	6.2 (12)	7.9 (6)	10.1 (27)	0 (21)
Average....	+			6.2 (32)	7.8 (30)	8.9 (22)	11.1 (39)	0 (33)
AHe/J	–			0.5 (10)	1.9 (8)	6.7 (5)	11.9 (6)	0 (7)
AKR/J	–			0.5 (7)	1.9 (7)	6.4 (7)	9.5 (5)	0 (2)
RF/J	–			0 (4)	1.2 (4)	5.7 (4)	8.7 (2)	0 (2)
SWR/J	–			0.5 (5)	2.0 (5)	6.8 (3)	10.0 (2)	0 (2)
Average....	0			0.25 (21)	1.7 (24)	6.4 (21)	10.0 (15)	0 (13)

The figures in parentheses indicate the number of mice used for each determination.

pearance of a blue area which could be readily seen and measured on the under side of the skin. The size of the blue area increased as a function of the quantity of antiserum injected into the skin. With large doses of antibody (25 μg N), lesions reaching 11 to 13 mm could be observed both in animals which did, and did not, possess MuB1. However, if smaller doses of antibody were injected, a difference between the sizes of lesions of negative and positive animals was discernible. This difference increased if the amount of antibody injected intradermally was decreased. At low doses of antibody, animals lacking MuB1 did not show lesions, whereas animals possessing MuB1 showed lesions of a diameter of about 5 mm (Table I, Fig. 1). It was thus possible to establish a correlation

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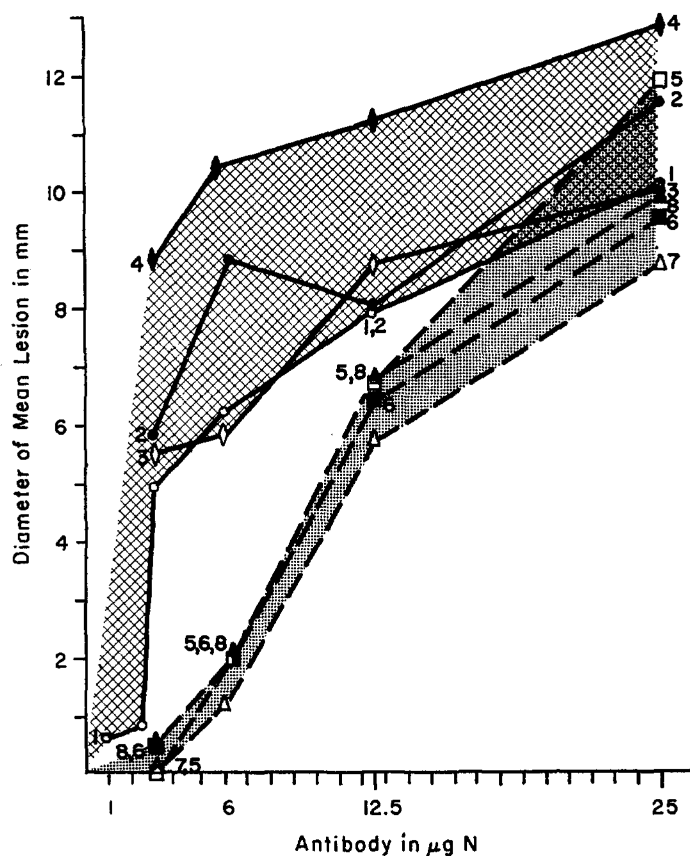

FIG. 1. A comparison of the reactivity of MuB1-positive and MuB1-negative mice; relation between the size of the "blued" area and the amount of antibody injected intradermally.——, response of MuB1-positive mice (all included in cross-hatched area); – – –, response of MuB1-negative mice (all included in dotted area).
Male mice (6 weeks old):
○, Swiss (Connaught) (*1*)
●, BALB/cJ (*2*)
◇, BUB/Bn (*3*)
◆, SJL/J (*4*)
□, AHe/J (*5*)
■, AKR/J (*6*)
△, RF/J (*7*)
▲, SWR/J (*8*)

between the presence and absence of MuB1 and the absence or presence of a blued area when 3 μg N antibody was injected intradermally and by the size of the blued area if 6 μg N antibody was injected intradermally. The question now arose whether the permeability of MuB1-negative animals would be af-

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fected if they were supplied with serum from MuB1-positive animals. MuB1-negative animals were injected intradermally with antibody (3 μg N) and 3 hours later with a mixture of antigen, Evans blue, and serum from MuB1-positive animals. Under these conditions, "blued" areas were observed. Blued areas did not appear if serum from animals lacking MuB1 was injected intravenously (Table II).

We have thus demonstrated that a humoral factor is involved in the increase of permeability at low doses of intradermally injected antibody.

So far, we had only been able to experiment with white mice. On the basis of the last mentioned experiment, it was possible to demonstrate presence or absence of MuB1 in dark skinned animals in which tests based on a direct reading of skin lesions would be difficult.

It will be seen from Table III that the serum obtained from eleven different strains which were MuB1-positive led to the appearance of positive PCA reactions in normally non-reactive AKR/J mice and that injection of the serum of MuB1-negative strains would not lead to any demonstrable increase in permeability.

We investigated whether the humoral factor could be supplied by sera other than those of mice and in particular, by those of guinea pigs. MuB1-negative mice (AKR/J) received intradermal injections of antibody (6 μg N) and 3 hours later intravenous injections of Evans blue, antigen, and various quantities of fresh, normal sera. The size of the blued area depended on the quantity and source of the fresh normal sera. Sera from guinea pigs were more effective than those of mice under the conditions of our experiment. Guinea pig sera were approximately 5 times as effective as those of Swiss mice (Table IV, Fig. 2).

We have so far shown that there is a considerable difference in the extent of the lesions occurring in PCA in animals possessing MuB1 and in animals lacking this antigen; an MuB1-negative animal can react like a MuB1-positive animal if supplied with a sufficient quantity of MuB1-positive serum and the humoral factor responsible can be supplied with guinea pig serum as well as with mouse serum.

Having established the involvement of a humoral factor in permeability changes, caused by antigen-antibody complexes, we turned to the problem posed by the marked change in the differences of the blued area of positive and negative animals as a function of the quantity of intradermally injected antiserum. This difference is very small if relatively high doses of antiserum are injected (Fig. 1).

It seemed possible that the heat-treated antiserum contained a heat-stable component, other than antibody, which affected permeability. At high doses, but not at low doses of antibody, enough of this substance might be injected to affect permeability profoundly. To test this assumption, the effects of low

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TABLE II

The Effect of Intravenously Injected Fresh Serum on PCA Reactions in MuB1-Negative Mice (AKR/J Male, 6 Weeks Old)

Source of serum			Amount of serum injected intravenously	Diameter as a function of μg N antibody injected	
Strain	MuB1	Sex		3 μg N	6 μg N
				mm	*mm*
A/HeJ	−	♂	0.125	0	0
		♂	0.089	0	0
		♀	0.089	0	0
AKR/J	−	♂	0.125	0	0
		♂	0.052	0	0
		♀	0.125	0, 0	0, 0
BALB/cJ	+	♂	0.025	10, 10.5	11, 13.5
CBA/J	+	♂	0.025	6, 9.5	9, 11.5
CE/J	−	♀	0.125	0	0
C57BR/cdJ	+	♂	0.125	6.5, 5	10, 8.5
		♂	0.025	12	14
		♀	0.025	8	10.5
C57L/J	+	♂	0.025	5, 6	8.5, 9.5
		♀	0.025	4	8
C58/J	+	♂	0.025	6	6
DBA/2DeJ	−	♂	0.052	0	0
MA/J	+	♂	0.025	4	10
PL/J	+	♂	0.025	10	13.5
RF/J	−	♂	0.125	0	0
		♀	0.125	0	0
		(Pool) ♂	0.125	0*	0*
Swiss	+	♂	0.025	0‡	0‡
				7.5	16
SWR/J	−	♂	0.125	0	0
		♂	0.073	0	0
		♀	0.059	0	0
SL/R1	+	♂	0.025	5	5.5
129/J	+	♂	0.073	4.5	4
		♀	0.125	6	7.5
C57BL/6J	+	♂	0.025	10, 11, 12.5	12.5, 15, 18.5

* 6 determinations.

‡ 8 determinations.

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quantities of intradermally injected antibody were compared when the antibody was diluted with 0.15 M NaCl and when it was diluted with normal heat-treated (56°C, 1 hour) rabbit serum. It was found (Table V) that the presence of normal heat-treated antiserum did not affect the size of the blued area. We therefore could eliminate the possibility that a substance in heated rabbit serum, other than antibody, contributed to the blueing. We considered next, the possibility that two types of independent reactions occurred of which one did not involve the complete hemolytic complement system. To investigate this possibility we examined the size of the blued area when antigen was injected

TABLE III

Summary of the Effect of Fresh Serum on PCA Reactions in MuB1-Negative Inbred Strain AKR/J Male, 6 Weeks Old

Total No. of strains	Total No. of sera	MuB1	Antibody injected	PCA reactions		
				Absent	Present	Mean diameter
			μg N			*mm*
11	21	+	3 6	0	11	7.5 10.5
6	14	−	3 6	14	0	0 0
Saline, 0.15 M	—	—	3	6	1	0.5
NaCl			6	4	3	1.9

intravenously *before* the injection of antibody, in other words, when there was no "latent" period and under conditions which according to Fischel and Kabat (13), Levy (16), and Benedict and Tips (35), result in the passive reversed Arthus reaction. Under these conditions no difference was found in the size of lesion elicited in animals possessing, and in animals lacking, MuB1, as can be seen from the data presented in Table VI.

Thus, if antigen and antibody were injected at the same time there was no difference in the size of the blued area of MuB1-positive and negative mice. On the other hand, a marked difference between the blued areas of these two types of animals was observed when antigen was given 3 hours after the injection of antibody. It therefore seemed that permeability changes occurring when antigen and antibody were injected simultaneously did not require the complete hemolytic complement system, whereas the permeability changes occurring after antibody had been in the skin tissue for 3 hours depended on the presence of the complete hemolytic complement system. To study this further, we de-

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TABLE IV

Effect of Intravenous Administration of Varying Amounts of MuB1-Positive Serum on the Extent of PCA Reaction in MuB1-Negative Mice

Strain AKR/J male; amount of antibody injected: 6 μg N.

Source of serum			PCA reaction, diameter	
Species	Strain	Amount of serum injected intravenously	Individual measurements	Average of all measurements
			mm	*mm*
Mouse	Swiss	0.125	9, 21, 8.5, 11, 10.5, 13	12.2
		0.025	9, 12, 16, 9.2, 9, 7.5, 7	12.0
		0.005	6.5, 8.5, 4, 5.5, 5.5	7.5
		0.001	5.5, 6, 5, 4, 3	4.7
		0.0007	3.5, 2.5, 4.5, 3.7, 3, 0, 0, 0	2.2
		0.0005	0, 0, 0, 3.7	0.9
		0.00025	0, 0, 0, 3.5	0.9
	Swiss, heated 56°C, 20	0.125	0, 0, 0, 5.5, 7, 3.5	2.7
	C57BR/cdJ	0.125	10, 8.5	9.2
		0.042	4, 8	6.0
		0.125	10.5	
		0.05	7	
		0.025	7, 10.5	8.80
		0.0125	0, 0	
		0.0062	0, 0	
Guinea pig	Hartley, inbred	0.025	Generalized reaction	∞
		0.005	15, 12, 13, 10.5	12.6
		0.001	9, 6.5, 7.5, 7.5, 8, 7	7.5
		0.0005	9, 7.5, 8, 7.5	8.0
		0.00035	7.5, 6, 7.5, 5, 5	6.2
		0.00025	0, 0, 7, 7.5, 8, 7, 6.5, 5	5.1
Control	Saline		0, 3.5, 0, 0, 4.5, 5, 0	1.9

termined the size of the blued area as a function of the time elapsing between intradermal injection of 3 μg N antibody and intravenous injection of antigen. This was tested in AKR/J mice which lack the antigen MuB1 and with MuB1-positive Swiss mice. In MuB1-negative mice, the size of the blued area was greatest when intradermal and cutaneous injections were given simultaneously, was small when the interval between injections was increased beyond 30 minutes, and could not be observed with an interval of 1 hour (Table VII). In the MuB1-positive animals, on the other hand, the blued area was still very large

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if the interval between injections was 3 hours and remained detectable for intervals of up to 6 hours.

The question now arose whether the length of time for which this first reac-

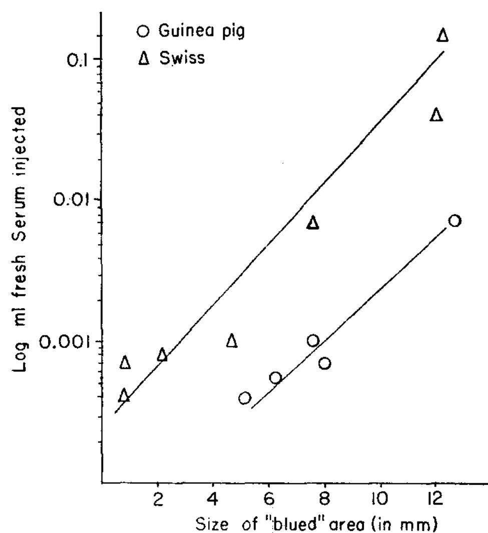

FIG. 2. The relation between the size of the blued area and the quantity of fresh, normal sera injected into MuB1-negative mice (AKR/J). ○, guinea pig sera; △, sera from MuB1-positive Swiss mice.

TABLE V

Effect of Serum Diluent on PCA Reaction at Low Concentration of Antibody

Strain AKR/J male, 6 weeks old; ribonuclease serum 801 injected intradermally: 3 μg N antibody; NRS: normal rabbit serum inactivated (56°C, 1 hour). (Same pool as used in NRS controls injected intradermally.) Ribonuclease antigen in saline.

Strain	Serum 801 diluted in	Diameter	Mean diameter
		mm	*mm*
AKR/J	NRS	0, 0, 0, 0, 0, 0, 0, 4	0.5
	0.15 M NaCl	0, 0, 0, 0, 0, 0, 3.5	0.5

801 dilution: 0.1 ml; 801S 1.0 mg N antibody/ml. 0.7 ml; diluent (NRS or 0.15).

tion, not requiring hemolytic complement, can be observed, depended on the quantity of antibody injected intradermally.

To investigate this possibility, we examined the size of the blued area as a function of the time elapsing between intradermal injection of 25 μg N antibody and intravenous injection of antigen. This was tested in AKR/J mice which lack the antigen MuB1 and in Swiss mice which possess MuB1.

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At high doses of antibody the size of the blued area underwent little change if the interval between injections was increased from 0 to 10 hours. If the interval was further increased a marked difference in the blued area of MuB1-positive and negative animals was observed and at 15 hours no permeability

TABLE VI

Size of the Blued Area when Intravenous Ribonuclease Injection Preceded Intradermal Antibody Injection

Strain	MuB1	Serum	Concentration	Diameter of the reaction, individual measurements	Mean diameter
				mm	*mm*
AKR/J	–	NRS	Undil.	0, 0, 0, 0, 0, 0	0
			1/20	0, 0	0
			1/80	0, 0	0
		801 RnS	Undil.*	12.5, 12.5, 10.5, 11	11.6
			2.5 μg N antibody	6.5, 7	6.7
			1.25 μg N antibody	7.5	7.5
			0.6 μg N antibody	4, 5, 6	5
			0.3 μg N antibody	0, 0	0
			0.15 μg N antibody	0, 0, 0, 0	0
SJL/J	+	NRS	Undil.	0, 0, 0, 0, 0, 0	0
			1/20	0, 0	0
			1/80	0, 0, 0	0
		801 RnS	Undil.*	10.5, 10, 10, 11	10.4
			2.5 μg N antibody	6, 6.5	6.25
			1.25 μg N antibody	6.5, 8.25	7.3
			0.6 μg N antibody	4, 6, 7, 5.5	5.6
			0.3 μg N antibody	0, 0	0
			0.15 μg N antibody	0, 0	0
Swiss	+	NRS	Undil.	0, 0, 0, 0	0
		801 RnS	25 μg N antibody	9, 9. 5, 8. 25, 9.25	9

Conditions of the experiment: Intravenous injection of Rnase in Evans blue (0.25 ml). Final concentration of ribonuclease: 100 μg N; final concentration of Evans blue: 0.5 per cent. Immediately after intravenous injection, intradermal injection of serum dilutions (0.025 ml). Reading: inner side, 1 hour after intradermal injection.

* 25 μg N.

increase could be seen in MuB1-negative animals. At the same interval the blued area of MuB1-positive animals was still 70 per cent of that obtained when injections were given simultaneously. As the interval was increased beyond 15 hours the size of the blued area decreased but was still demonstrable after 24 hours.

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TABLE VII

Size of the Blued Area as a Function of the Time between Intradermal Injection of Antibody and Intravenous Injection of Antigen (Latent Period)

Strain males	MuB1	Latent period	Mean diameter as a function of μg N antibody injected intradermally			
			3 μg N antibody		25 μg N antibody	
		min.	*mm*		*mm*	
AKR/J	–	0	5.5, 7, 5.75, 5.5, 7.5, 6, 5.5, 6, 5.5, 6	(6.0)	12.5, 12.5, 10.5, 11	(11.6)
		0.5–1	5.25, 5.5, 5.5, 5.25, 5.5, 5.25, 4.75, 6.5, 6, 5.5	(5.5)	7.5, 7.5	(7.5)
		30	5.25, 5.25, 0, 5.5, 5.25, 5.5, 6.75, 6.5, 4, 6.25	(5.0)	10, 9	(9.5)
		45	4.5, 5.5, 7.5, 7.5, 5.25, 4.5, 3.5, 3.5	(5.3)	9, 9.5	(9.3)
		60	3.5, 3, 4.75, 5, 4, 2, 2.5, 0, 0	(2.8)	6, 6	(6.0)
		90	2, 0, 0, 0, 4, 4.25, 0, 0, 0, 2	(1.2)	7.25, 6.5	(6.4)
		120	0, 0, 0, 0, 0, 0, 0, 0, 0, 0	(0.0)	7, 6.5	(6.8)
		180	0, 0, 0, 3.5, 4.5, 5	(2.1)	9, 8, 8.5, 11.5, 10.5	(9.5)
		600			8, 6, 6, 8, 5, 6, 8, 6	(6.6)
		915			0, 0, 0, 0, 0, 0, 2, 2	(0.5)
		1440			0, 0, 0, 0, 0, 0, 2, 2	(0.5)
BALB/cJ	+	180	4, 6, 5, 7, 7	(5.8)	12, 11	(11.5)
		1200			6, 4.5, 5.5, 4, 5, 7	(5.3)
Swiss	+	0	5.75, 6, 8.5, 7, 6, 6, 0, 4.5, 5.25, 6, 6.5, 7	(5.7)	9, 9.5, 8.25, 9.25	(9.0)
			5.5, 6.5, 7.5, 7.75, 3.5, 6, 4, 4.25, 4.75, 5.5, 6, 5.75	(5.6)		
		90	5, 5.25, 6.5, 6, 5.5, 4.5, 0, 4, 5.25, 4, 4.5, 4.75	(4.6)		
		180	6.5, 5, 3.75, 4.75, 4, 5, 5.75, 5.5, 5.25, 3.25	(4.8)	9.5, 10, 11.5, 8.5, 11	(10.1)
		225	0, 0, 3.5, 6.5, 0, 0, 3.5, 3, 10, 6.5, 5, 5.5	(3.6)		
		270	4, 5, 0, 0, 4.5, 5.5, 5.75, 6, 4.5, 4, 0, 0	(3.3)		
		360	0, 0, 0, 0, 0, 0, 5.75, 4.5, 0, 0, 0, 4.25	(1.2)		
		420	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	(0.0)		
		600			11.5, 11.5, 10, 10	(10.8)
		915			5.75, 6, 9.5, 10.25, 8, 8, 10, 10	(8.4)
		1440			0, 0, 0, 0, 0, 5, 5, 5.5	(1.9)

Numbers in parentheses are the means of all determinations.

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It would thus seem reasonable to conclude that two different reactions are involved in permeability increase. The first reaction, occurring when antigen and antibody are injected simultaneously, does not require the complete

TABLE VIII

The Effect of Fresh Serum on PCA in MuB1-Negative Mice (AKR/J Male, 6 Weeks Old) when the Interval between Intradermal Injection of Antibody and Intravenous Injection of Antigen Was 15 Hours

Source of serum		Amount of Serum injected intravenously	Diameter, 25 μg N antibody injected intradermally
Strain	MuB1		
		ml	*mm*
Swiss	+	0.125	8, 7, 6.5, 7, 6.5
AKR/J	−	0.125	0, 0, 0, 0, 0, 0
0.15 M NaCl	−	—	0, 0, 0, 0, 0, 0, 2, 2

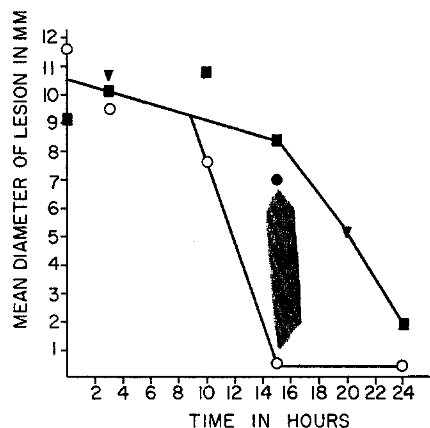

FIG. 3. The effect of the interval between the intradermal injection of antibody and the intravenous injection of antigen on the size of the blued area. A comparison between MuB1-negative and MuB1-positive animals.

Animals were injected intradermally with 25 μg N antibody and at various periods thereafter were given intravenous injections of the antigen. The arrow indicates the change in the diameter of the blued area when MuB1-negative animals were injected with fresh normal serum of MuB1-positive mice.

Size of blued area in: ■, Swiss (MuB1-positive); ▼, BALB/cJ (MuB1-positive); ○, AKR/J (MuB1-negative); ●, AKR/J when serum from MuB1-positive Swiss mice is injected intravenously at the same time as the antigen.

hemolytic complement system. The reaction occurring when antigen is injected some time after the antibody requires the complete hemolytic complement system. The interval for which the first of these two reactions is demonstrable depends on the quantity of antibody injected. If MuB1-negative animals were injected with 3 μg N antibody, the contribution of the first reaction was negli-

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gible 3 hours after the injection of the antibody, but was still very marked when 25 μg N antibody had been given.

The difference between the reaction of MuB1-positive and MuB1-negative animals appeared to be attributable to a hemolytic complement–requiring second reaction which did not occur in MuB1-negative animals. If this conclusion is correct, it should be possible to produce a blued area in MuB1-negative animals even 15 hours after the injection of 25 μg N antibody by providing the humoral factor required by the second reaction.

To test this latter conclusion, MuB1-negative animals were injected intradermally with 25 μg N of antibody and were injected 15 hours later with antigen mixed with fresh serum from MuB1-positive mice. The MuB1-negative animals, so injected, showed a blued area, comparable in size to that of MuB1-positive mice, injected under similar conditions, but not given additional mouse serum. If MuB1-negative mice were injected with sera from MuB1-negative animals, a blued area did not appear (Table VIII, Fig. 3). Thus, if large doses of antibody are given to complement-deficient mice, a blued area is not observed if antigen is given 15 hours later, unless a missing complement factor is supplied.

DISCUSSION

The detailed analysis of *in vivo* reactions is aided by the availability of experimental animals which differ in molecularly defined factors. The availability of complement-deficient mice offers new possibilities in this respect and we have utilized such animals in the investigation of the early stages of immunological injury.

Two phases in the permeability reaction of the mouse are demonstrable; the first of these reaches its peak shortly after the injection of antigen and does not require the complete hemolytic complement system. The second, later phase, requires the complete hemolytic complement system since it does not occur in MuB1-negative mice.

The duration of the two phases, but not their relative difference, depends on the quantity of antibody injected, so that an interval can be found for small or large doses, at which MuB1-positive animals show blueing, and MuB1-negative animals do not respond. If very large doses of antibody are injected (25 μg N), the same two phases were observed but their duration was greatly increased. The first phase became negligible only after 15 hours. If, after this interval, normal serum from MuB1-positive animals is administered to MuB1-negative animals, a lesion develops and differs little in size from the lesions of MuB1-positive animals. It would, therefore, seem justified to conclude that a humoral factor is involved. Since this humoral factor can, in the second of the two phases, be supplied with the serum of MuB1-positive but not with that of MuB1-negative mice (29, 31), it is reasonable to conclude that the humoral factor is connected with the complete hemolytic complement system.

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What mechanism mediates the permeability increase in the first phase of the permeability reaction, the only one seen in MuB1-negative animals, is not clear. Such animals, while lacking one component of complement, which may be C'3 and possibly C'3d (28, 36), still seem to possess most of the other components of complement. Whether this incomplete complement system can bring about permeability increase or whether an alternative system operates in the blueing reaction of MuB1-negative mice must remain an open question.

The differences in the complement system of MuB1-negative and MuB1-positive mice, alone, cannot explain the difference in the cutaneous reaction of the two types of mice. Heterogeneity in the interaction between complement and antibody must also be involved. The first of the two reaction phases cannot involve all antibody molecules, interacting with two different systems which both promote permeability increase, since this would result in marked differences in the cutaneous response of MuB1-positive and MuB1-negative animals, irrespective of the interval between injections. However, the permeability increase, observed in MuB1-negative and MuB1-positive mice, was similar and seemed independent of the presence or absence of the hemolytic complement system, if antigen was injected prior to antibody (Table VI).

The two phases in the cutaneous response of the mouse may be the result of a heterogeneity of the antibody such as that described in guinea pigs (7, 10). An immune serum may contain two, and possibly several fractions of antibody, which may differ in the rate at which they fix to tissues, combine with antigen, and fix complement. A fraction of antibody which fixes rapidly to tissues and affects permeability through some such component as C'1 or through a system other than complement, may cause the first of the two phases observed in the cutaneous response of the mouse. A fraction of antibody which fixes slowly to tissue and involves activation of one of the terminal factors of the hemolytic complement system, may be responsible for the second phase of the blueing reaction.

An alternative explanation of the two observed phases of the cutaneous reaction, also possibly connected with heterogeneity of antibody, would attribute the two phases of the cutaneous response to the effect of antigen-antibody complexes not fixed to cells, on the one hand, and of cell-fixed complexes, on the other hand. Antigen-antibody complexes, not fixed to tissue, lead to increased permeability. If such complexes persisted for a sufficiently long time, they might contribute to the increase in permeability, resulting from the combination of antigen with fixed antibody. Thus two reactions would occur simultaneously; the first, due to unfixed antigen-antibody complexes, possibly corresponding to the initial stages of the RPA reaction, would be most intense shortly after the intradermal injection of antibody and would decrease as a function of time elapsed after the intradermal injection of antibody. The second, the reaction of PCA, would, at first, increase with time elapsed after intracutaneous injection of antibody and would subsequently decrease.

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At present, a decision between the above alternatives cannot be made, the nature of the antibody heterogeneity remains to be explored and only the study of different antibody preparations and of fractions of antibody will allow us to decide between the mechanisms discussed above.

In summary, we are led to the conclusions that early immune damage is caused by two distinct reactions which differ in their time course, and of which only one involves the complete hemolytic complement system, and that a heterogeneity of the antibody with respect to tissue fixation and/or interaction with complement is responsible for the occurrence of two phases of the cutaneous response.

Contradictory results, obtained by different investigators of the role of complement in immune damage, may be attributable to differences between different mammalian species, and to variations in the same species in the concentration of antibody fractions which differ in their ability to fix to tissues and to fix complement. Furthermore, difficulties in separating the distinct but overlapping cutaneous reactions, demonstrated in the foregoing experiments, may result in apparently contradictory findings.

SUMMARY

Intradermal injection of mice with ribonuclease antibody, followed by intravenous injection with ribonuclease, resulted in permeability increase, demonstrable by "blueing." The size of the blued area depends on the quantity of antibody injected and on the interval between the two injections. If antigen was injected first and antibody was injected subsequently, a similar increase in permeability was observed in animals having a complete complement system (MuB1-positive) and in animals which have a deficient complement system (MuB1-negative). Marked differences in response were observed between these two types of mice if antigen was injected some hours after the antibody. In MuB1-negative mice, a blueing reaction was not observed at intervals between injections (2½ hours if 3 μg N antibody and 15 hours if 25 μg N antibody were injected intradermally) at which MuB1-positive animals showed a marked permeability increase. At these intervals, blueing did occur in MuB1-negative animals if they were injected with the serum of MuB1-positive mice or with fresh guinea pig serum. Blueing was not induced if the serum of MuB1-negative mice or heated guinea pig serum was injected. The occurrence of two distinct phases of the cutaneous reaction, of which only one involves the complete hemolytic complement system, was deduced from these observations.

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