Immunology, 1965, **9**, 303.

The Effect of Decomplementation on Delayed-Type Hypersensitive Reactions to a Conjugated Antigen in Rats

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(*Received 16th November* 1964)

Summary. A method has been described for quantitative measurement of the intensity of cutaneous delayed hypersensitive reactions in rats. This method is based on the measurement of the weight of skin infiltration at the site of cutaneous reaction.

In vivo decomplementation of rats with antigen–antibody complexes or aggregated γ-globulin is accompanied by a decrease in delayed hypersensitivity responsiveness.

When the level of serum complement is reduced by 90 per cent delayed hypersensitive reactions are completely suppressed.

The results presented, although suggestive, do not provide a definite proof of the role of complement in the development of delayed hypersensitive reactions in the skin.

INTRODUCTION

Participation of complement (C′) in the mechanisms responsible for different types of hypersensitivity has been extensively investigated with different experimental techniques. A general review of the subject has been published recently by Osler (1961).

A useful tool for investigation of hypersensitivity has been provided by study of *in vivo* reduction of C′ level (decomplementation) on the manifestation of hypersensitive reactions. *In vivo* decomplementation may be achieved by absorbing C′ either on antigen–antibody complexes or on aggregated γ-globulins given by a single injection. The reduction of serum C′ level obtained by such a method is of short duration, and so it has only been applied to the study of C′ participation in hypersensitive reactions of the immediate types (anaphylaxis and Arthus type hypersensitivity) (Bier, Siqueira and Osler, 1955; Osler, Hawrisiak, Ovary, Siqueira and Bier, 1957; Osler, Randall, Hill and Ovary, 1959; Christian and Thurer, 1962; Frick, Stiffel and Biozzi, 1962; Weigle, 1961; Bier 1962). Preliminary experiments in rats have shown that the C′ level of serum can be kept reduced *in vivo* for a period of 24 hours by repeated injections of either antigen–antibody complexes or human γ-globulin aggregates.

The comparatively low C′ concentration in rat serum and the high resistance of this animal species to the shocking effect of the decomplementing agents, provide favourable conditions for a pronounced, long lasting, depletion of C′ allowing the study of *in vivo* decomplementation on hypersensitive reactions of the delayed type. Recently Flax and

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Waksman (1962) described typical cutaneous hypersensitive reactions of delayed type in rats sensitized with serum proteins in complete Freund's adjuvant.

The present investigation was undertaken to study the effect of *in vivo* decomplementation on delayed cutaneous reactions in rats sensitized with a conjugated antigen according to the method of Flax and Waksman (1962).

Delayed cutaneous hypersensitive reactions in rats are characterized by diffuse subcutaneous cellular infiltration. In view of the deep location of the infiltration it is difficult to appreciate the exact size of the reactions. Mild and small reactions may often be undetectable by simple inspection and palpation. To overcome this difficulty, a method has been devised to allow precise definition of the intensity of cutaneous reactions by the quantitative measurement of the skin infiltration. The degree of local skin infiltration was measured by taking the difference between the weights of the skin with the reaction and that of a piece of normal skin of the same size cut from the symmetrical contralateral side.

MATERIAL AND METHODS

Experiments were carried out on adult 'Wistar' and 'Long Evans' rats of both sexes weighing about 300 g.

Sensitization

Rats were injected in each hind foot pad with 0·1 ml of an emulsion prepared by mixing equal volumes of complete Freund's adjuvant and saline containing 7 mg/ml of picrylated egg albumin (P.Ea) prepared by the method of Benacerraf and Gell (1959).

Measurement of the intensity of delayed cutaneous reactions

Rats were challenged by intradermal injections of 50, 100 and 150 μg of P.Ea in 0·1 ml saline on the right flank at the periods after sensitization shown in Table 1.

The trunk of the animal was shaved before the experiment with an electric clipper, and care was taken not to damage the skin. Animals were inspected, 4, 6 and 8 hours later, to detect whether immediate hypersensitive reactions occured. Twenty-four hours after challenge rats were killed by bleeding and the weight of the skin in the area of the reaction was determined as described below. The sites of the skin reaction were identified with a skin marker and the median dorsal and ventral lines were drawn. The skin of the trunk was removed after careful separation from the muscular layer. It was symmetrically folded in half along the median dorsal line, stretched and fixed with pins on a piece of soft wood.

A series of eight sharp punches varying from 8 to 25 mm in diameter were used for cutting identical sections from the layers of skin. A punch slightly larger than the area of infiltration was selected for the purpose. In this way it was possible to obtain the disc of skin involved in the reaction together with a symmetrical piece of underlying control skin from the opposite side. The two discs were immediately weighed and the extent of skin infiltration was established by substracting the weight of the normal from that of the infiltrated skin disc.

The number of rats used in the experiments are shown in the tables, and results are expressed as the mean values for the weight differences.

Measurement of circulating antibody to P.Ea

Blood samples of 0·2 ml were drawn from the retro-ocular venous plexus (Halpern and Pacaud, 1951) and pooled. The serum was separated after clotting and the antibody titre determined by passive agglutination of rabbit red cells coupled to P.Ea with benzidine

according to the technique described by Halpern, Jacob, Binaghi and Parlebas (1961). The antibody titre was expressed as the highest dilution of serum giving positive haemagglutination.

Titration of C′

Complement titration was performed according to the method of Rosenberg and Tachibana (1962) by establishing the volume of rat serum giving 50 per cent haemolysis of sheep erythrocytes optimally sensitized with rabbit antibody. The dosage of haemoglobulin was determined in a Beckman-Spinco electrophotometer with a cell of 0·1 ml. Serum for C′ titration obtained from blood freshly drawn from the retro-ocular vascular plexus, was immediately cooled in crushed ice. Equal volumes of individual serum were pooled for C′ titration, and so each C′ titre represents the mean value for the group of rats under examination.

Methods of decomplementation of rats in vivo

In vivo decomplementation was performed by absorbing C′ on antigen–antibody complexes or aggregated human γ-globulin.

Antigen–antibody complexes. Rabbit immune serum against bovine serum albumin (anti-BSA) titrating 1·100 μg AbN/ml was used. Rats were injected intravenously with 500 μg N of anti-BSA. This dose was repeated five times at the intervals indicated in Fig. 1. Each dose of anti-BSA was immediately followed by the injection of 250 μg N of BSA. The first injection of BSA was given intravenously the subsequent ones intraperitoneally. Control rats received equivalent volumes of normal rabbit serum instead of rabbit anti-BSA, followed by BSA at the same doses and by the same route as decomplemented animals. Both immune and normal rabbit serum were decomplemented by heating at 56° for 30 minutes.

Aggregated human γ-globulin (AHGG). AHGG was prepared according to the technique of Christian and Thurer (1962). The first injection of 3 mg N of AHGG was given intravenously 30 minutes before challenge. The same dose was repeated four times by the intraperitoneal route at the intervals indicated in Fig. 1.

White blood cell counts

Total and differential leucocyte counts were carried out before decomplementation and repeated at the times indicated in Table 3. Blood drawn from the retro-ocular vascular plexus was collected in heparinized tubes. Equal volumes of individual blood samples were mixed. Total white cell counts were made in a Malassez's cell and percentages of the various types of leucocytes were established from smears stained by the May–Grünwald–Giemsa method.

RESULTS

The method for measuring the extent of the skin reactions allowed the estimation of small degrees of hypersensitivity in terms of weight of skin infiltration. Preliminary assays in normal rats showed that the difference in weight between symmetrical skin discs was less than 5 per cent of the lighter of the pair. Differences below 5 per cent were therefore considered as non-significant, and were not taken into account for the calculations.

Table 1 shows the intensity of skin reactions in Long Evans and Wistar rats challenged with 100 μg of P.Ea at different times after sensitization. For Long Evans rats corresponding titres of circulating antibody are also indicated.

TABLE 1

INTENSITY OF DELAYED CUTANEOUS REACTIONS, MEASURED BY THE WEIGHT OF SKIN INFILTRATION, IN RATS CHALLENGED WITH 100 μg OF P.Ea AT DIFFERENT TIMES AFTER SENSITIZATION

Strain of rats	No. of rats	Days after sensitization	Mean weight of cutaneous infiltration produced by the injection of 100 μg of P.Ea (mg)	Mean titre of circulating antibodies
Long Evans	4	6	0	1 : 1536
	4	9	345	1 : 6500
	5	15	330	1 : 32000
	5	30	350	1 : 255000
Wistar	4	10	0	
	5	17	272	
	5	22	372	

Reactions reached maximal intensity at the ninth day in Long Evans rats while in Wistar rats a corresponding intensity was only obtained after 22 days. For this reason, the effect of decomplementation was studied 9 days after sensitization in Long Evans and 22 days in Wistar rats in all the subsequent experiments.

The titre of circulating antibody was relatively low (1 : 6500) at the ninth day when cutaneous delayed reactions reached their maximal intensity. At this time reactions became apparent 8–10 hours after antigen injection and increased progressively up to the twenty-fourth hour. In animals tested at the fifteenth, and particularly at the thirtieth day an immediate type reaction occured 2–3 hours after challenge. This early reaction was characterized by oedema and small central haemorrhagic patches, but not erythema, in contrast to the delayed erythematous non-haemorrhagic infiltration observed at the ninth day. In many of the Wistar rats challenged at the twenty-second day both early and delayed types of lesion occurred; other rats gave only delayed reactions.

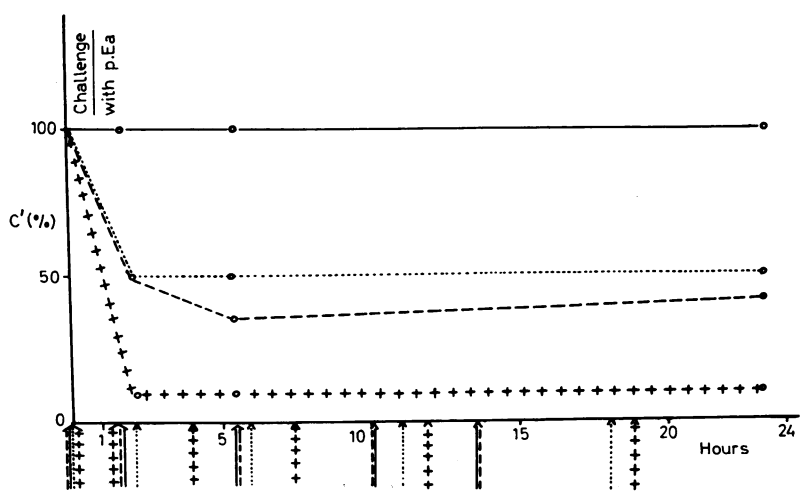

FIG. 1. Reduction of the serum complement titre in rats repeatedly injected with antigen–antibody complexes or aggregated human γ-globulin (AHGG). ———, Rabbit normal serum + BSA; — — —, rabbit anti-BSA+BSA (Long Evans rats). HGG aggregate: + + +, Wistar rats; ·····, Long Evans rats. The arrows indicate the time of injections

Fig. 1 shows the degree of C′ reduction in serum of rats treated with antigen–antibody complexes or AHGG. Control animals receiving normal decomplemented rabbit serum together with BSA showed no significant changes in C′ level. A C′ depletion of about 50 per cent was obtained in Long Evans rats treated with AHGG 90 minutes after the first decomplementing injection. This level could be maintained up to the twenty-fourth hour by repeated injections of AHGG. A similar, somewhat stronger effect was produced by antigen–antibody complexes, a reduction of 60 per cent of C′ initial titre being obtained after the fifth hour. In Wistar rats the injection of antigen–antibody complexes produced a drastic reduction of C′ serum titres which fell to 10 per cent of the initial titre. These could be kept at a low level during the next 24 hours by repetition of the decomplementing injections.

The inhibitory effect of decomplementation on delayed cutaneous hypersensitive reactions is demonstrated in Table 2.

TABLE 2

INTENSITY OF DELAYED CUTANEOUS REACTIONS IN CONTROL AND 'DECOMPLEMENTED' RATS

Strain of rats	No. of rats	'Decomplementing' treatment	C′ titre between 5 and 24 hours (%)	Mean weight of cutaneous infiltration elicited by the following concentrations (μg/0·1 ml) of P.Ea		
				50	100	150
Long Evans	10	None	100	185	361	295
	5	Normal rabbit serum+BSA	100	146	204	300
	5	BSA+anti-BSA	40	0	22	52
	5	AHGG	50	65	30	—
Wistar	5	None	100	90	372	—
	5	BSA+anti-BSA	10	0	0	—

Untreated rats, and those injected with normal rabbit serum and BSA, with normal C′ levels (100 per cent) developed skin reactions of about same intensity. The intensity of skin infiltration was proportional to the concentration of P.Ea used for eliciting the reaction. Decomplemented animals showed a consistant reduction in the intensity of skin reactions roughly proportional to the residual C′ level. The weight of local skin infiltration at the site of the delayed reaction elicited with 100 μg of P.Ea was about ten times smaller in decomplemented Long Evans rats than in controls.

Delayed skin hypersensitivity was completely abolished in decomplemented Wistar rats showing the strongest degree of C′ reduction (90 per cent). In these animals early oedematous reactions were also suppressed by decomplementation.

Another group of sensitized Wistar rats, decomplemented with antigen–antibody complexes and challenged with P.Ea in the same way showed a complete suppression of delayed skin sensitivity 24 hours after challenge, as judged by external examination of the skin sites. These animals were kept alive and tested again 4 days after the first challenge. At this time C′ titre had already returned to normal and rats showed delayed cutaneous reactions of normal intensity.

The data summarized in Table 3 show that *in vivo* decomplementation carried out according to the same techniques used for the study of delayed hypersensitivity did not significantly modify the number and the cell type of circulating leucocytes 6 and 23 hours after the first decomplementing injection.

TABLE 3

WHITE BLOOD CELL COUNTS AT DIFFERENT INTERVALS AFTER 'DECOMPLEMENTATION'

Treatment	Hours after challenge with P.Ea	Total white cells (counts/mm³)	Differential white cells (counts per cent)				
			Neutrophils	Eosinophils	Small lymphocytes	Large lymphocytes	Monocytes
None	0	12000	56	2	22	14	6
	5	9400	65	0	20	9	6
	23	12100	60	0	23	12	5
Normal rabbit serum+BSA	1·30	12400	58	0	23	14	5
	5·40	11100	60	0	21	13	6
	23	12000	54	0	27	13	6
BSA+anti-BSA	1·30	11800	63	1	19	11	6
	5·40	12400	60	0	22	11	7
	23	10000	49	1	28	16	6

DISCUSSION

The data presented above show a parallelism between C′ serum level and the ability of sensitized rats to respond with delayed hypersensitive reactions at the site of antigen injection. This fact, however, does not prove a causal relationship between C′ level and delayed responsiveness. The inhibition of delayed hypersensitivity may only be coincidental with the reduction of serum C′; it could also have been due to other modifications possibly produced by the decomplementing treatment.

The only well established fact in the pathogenesis of delayed hypersensitivity is that it appears to be mediated by sensitized cells (Boyden, 1958; Chase, 1959). These cells, probably small lymphocytes (Waksman, Arbouys and Arnason, 1961) are produced by local lymph nodes, and are already present in the systemic circulation when the antigen injection elicits the cutaneous reactions (Neveu, Biozzi, Halpern, Liacopoulos and Branellec, 1963).

The absence of any effect of the decomplementing treatment on the numbers of circulating leucocytes (Table 3) shows that suppression of delayed hypersensitivity was not produced by an effect on the sensitized cells.

The variability in the mechanisms of the same type of hypersensitivity in different animal species adds further difficulty in evaluating the general role eventually played by C′. This difficulty is illustrated by comparison of the results reported by various authors on the role of C′ in hypersensitivity of anaphylactic type in different animal species.

In vivo decomplementation reduces passive cutaneous anaphylaxis (PCA) in rats (Bier *et al.*, 1955; Osler *et al.*, 1957) but it is without action on PCA or systemic anaphylaxis in mice (Frick *et al.*, 1962). In guinea-pigs only PCA seems to be C′ dependant while passive systemic anaphylaxis is not (Christian and Thurer, 1962). Recent investigations have shown that anaphylaxis in guinea-pigs can be transmitted passively by homologous antibodies which do not fix haemolytic complement (Ovary, Benacerraf and Block, 1963; Block, Kourilsky, Ovary and Benacerraf, 1963).

For all these reasons, one should be very cautious in interpreting results of experiments carried out with the technique of *in vivo* decomplementation, as stressed by Bier (1962) in a critical analysis of the role of complement in immediate hypersensitive reactions. However,

the powerful inhibitory effect induced by decomplementing treatment is highly suggestive and deserves further investigation.

The observations of a delayed decrease in C′ serum concentration produced by tuberculin injection in guinea-pigs sensitized with tubercle bacilli (Rice, Boulanger and Koust, 1954; Morikawa, Okuyama and Tokita, 1957) are also consistent with the possible role of C′ in the mechanism of delayed hypersensitive reactions.

The suppression of early oedematous and haemorrhagic reactions of the Arthus type exhibited by the decomplemented Wistar rats challenged at the twenty-second day of sensitization is in agreement with present views on the intervention of serum C′ in Arthus type hypersensitivity (Weigle, 1961; Bier and Siqueira, 1959).

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