AN EFFECT OF ANTIGEN-ANTIBODY INTERACTION ON BLOOD COAGULATION*

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The prominence of thrombosis in the pathogenesis of the Arthus phenomenon (1, 2), the alterations in the clotting mechanism seen in experimental anaphylaxis (3), and the occurrence of "fibrinoid" in lesions generally considered to be due to hypersensitivity suggest that the interaction of antigen and antibody *in vivo* may in some manner initiate coagulation. Experiments to be reported here indicate that such an effect can, in fact, be demonstrated *in vitro* under appropriate conditions. Although the mechanism of this phenomenon is not yet clear, it seems worth while to report the basic observations because of their apparent pertinence to various immunopathological problems.

Materials and Methods

Antigens.—The antigens used were ovalbumin (2× crystallized, obtained from Worthington Biochemical Corp.), bovine gamma globulin and bovine serum albumin (obtained from Armour Laboratories), native dextran (kindly supplied by Dr. E. Hehre), and preparations of the somatic antigen ("lipopolysaccharide endotoxin") of *Escherichia coli* strain 0111:B4 (obtained from Difco Laboratories, Inc., Detroit). Rabbits were immunized against ovalbumin or the bovine proteins by repeated intradermal and/or intravenous injections of antigen in doses which ranged from 1 to 50 mg. Serum obtained from immunized and normal animals was stored in the frozen state and aliquots were inactivated by heating at 56°C. for a half-hour before use.

Coagulation Time Measurement.—Whole blood was obtained from normal and immunized rabbits by cardiac puncture and immediately distributed in 2.0 or 4.0 ml. amounts in 15 x 75 mm. siliconized glass test tubes. The tubes were kept at room temperature, and the end-point of coagulation was taken as that time at which no flow occurred upon inversion of the tube. The effect of various test materials on the coagulation time was determined by adding these materials in a total volume of 0.1 or 0.2 ml. to the tubes before addition of the blood.

Under these conditions the coagulation time of rabbit blood was usually prolonged to from 20 to 40 minutes as compared to 8 to 12 minutes in clean but non-siliconized glassware. This prolongation of clotting time permitted an evaluation of the coagulation-accelerating effect

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of various added test materials. In a few experiments the coagulation time was prolonged by other methods as well, such as by the addition of small amounts of heparin or by carrying out the determinations in the cold, but all of the experiments reported here were done with siliconized glassware as described above.

TABLE I

Effect of Bovine Gamma Globulin on Blood of Normal and Immune Rabbits

Source of blood	Rabbit No.	Coagulation time				
		Concentration of BGG (μg./ml.)				
		0 (NaCl)	0.05	0.5	5.0	50
		min.	*min.*	*min.*	*min.*	*min.*
Normal rabbits	1	42	44	45	42	43
	2	34	37	37	40	40
	3	34	33	38	38	35
	4	25	25	25	25	24
Average		33.7	34.7	36.3	36.3	35.5
Immunized rabbits	1	25	25	25	22	17
	2	18	12	12	12	11
	3	30	19	20	17	16
	4	18	14	11	11	5
	5	30	25	20	18	17
	6	18	20	14	12	11
	7	29	21	25	17	18
Average		24.0	19.4	18.4	15.6	13.6

Each tube contained 0.1 ml. antigen dilution or physiological saline solution, to which was added 2.0 ml. whole blood. Concentrations of BGG are expressed as the final concentration in the clotting mixture. Variations in the control-clotting time from rabbit to rabbit appeared to be related to the care and dispatch with which cardiac puncture and distribution of the blood was accomplished. The data illustrate the consistent coagulation-accelerating effect of BGG on the blood of specifically immunized rabbits.

EXPERIMENTAL

Effect of Addition of Antigen to Blood of Immunized Rabbits.—It had been observed earlier (4) that the addition of ovalbumin *in vitro* to whole blood from immunized rabbits resulted in the prompt and striking agglutination of the platelets and leucocytes in the preparation. In the present study it was found that, in the absence of anticoagulants, such preparations clot in a much shorter time than do the appropriate control preparations. The results of experiments with ovalbumin, bovine serum albumin (BSA), and bovine gamma globulin (BGG) were quite comparable, and are illustrated in Table I. The addition of these antigens did not appreciably affect the coagulation time of normal rabbit blood but produced marked and reproducible acceleration of clotting

of blood samples from specifically immunized rabbits. This effect was observed over a wide range of antigen concentrations, with amounts of BGG as little as 0.05 μg./ml. blood being sufficient to produce significant depression of the clotting time. An attempt was made to correlate the concentration of antigen required to produce platelet clumping with that necessary to cause

TABLE II

Effect of Mixtures of Bovine Gamma Globulin and Normal or Immune Serum on the Coagulation Time of Normal Rabbit Blood

Serum added	Rabbit No.	Coagulation time					
		Concentration of BGG (μg./ml.)					
		0 (NaCl)	0.005	0.05	0.5	5.0	50
		min.	*min.*	*min.*	*min.*	*min.*	*min.*
Normal rabbit serum	1	36	—	35	36	35	32
	2	34	—	33	33	36	27
	3	21	—	21	24	22	19
Antiovalbumin serum	1	21	—	19	18	20	20
	2	18	—	18	16	18	16
	3	20	—	20	21	18	18
Anti-BGG serum	1	33	—	23	16	20	16
	2	20	—	13	8	13	10
	3	21	16	13	10	—	—
	4	35	25	17	12	—	—
	5	26	12	11	8	—	—
	6	24	13	11	8	—	—
	7	33	26	20	20	15	14
	8	35	27	27	20	18	22
	9	30	20	17	14	14	13

Each tube contained 0.05 ml. antigen dilution and 0.05 ml. undiluted inactivated serum mixed together immediately before addition of 2.0 ml. whole blood from normal rabbits. The rabbit numbers refer to the normal donors of whole blood used as the clotting system in each series of determinations. Mixtures of BGG with normal rabbit serum or rabbit antiovalbumin serum were without effect, while the mixtures of BGG with anti-BGG serum consistently produced significant shortening of the coagulation time.

accelerated coagulation, but the spontaneous slow agglutination of platelets in control preparations made it impossible to accurately determine an end-point in antigen titrations.

Effect of Antigen-Antibody Mixtures on Normal Rabbit Blood.—In order to determine whether the observed effect was mediated by serum antibody, small amounts of serum from the immunized rabbits were mixed with antigen *in vitro* and whole blood from normal rabbits was then added as an indicator

system. Table II shows the results of a typical experiment with BGG and anti-BGG serum, and it can be seen that the antigen-antibody mixture produced a considerable coagulation-accelerating effect. This system permitted the titration of antiserum as well as antigen, and Table III shows that the phenomenon was demonstrable over a wide range of dilution of antiserum and of concentration of antigen.

In some experiments there was a suggestion of a prozone; for example, the data in Table III suggest that a concentration of BGG of 5 μg./ml. was more

TABLE III

Titration of Anti-Bovine Gamma Globulin Serum

Concentration of BGG	No. of rabbits	Coagulation time					
		Dilution of antiserum					NaCl
		1/40	1/80	1/160	1/640	1/2560	
μg./ml.		*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
50	3	16.0	15.7	16.4	20.3	23.7	28.7
5.0	3	11.3	12.3	11.7	12.3	19.0	28.7
0.5	2	14.0	15.0	15.5	17.5	18.0	26.0
0.05	2	25.0	28.0	29.0	29.0	27.0	31.5
0 (NaCl)	3	25.3	24.8	23.0	21.0	24.0	24.2

Each tube contained 0.05 ml. antigen and 0.05 ml. antibody, as in Table II. The concentrations are expressed as final concentrations in the clotting mixture, after addition of 2.0 ml. normal rabbit blood. The coagulation times shown are the averages of determinations performed with either two or three normal blood donors, as shown. Even the highest dilution of serum tested showed some activity.

effective than 50 μg./ml. The investigation of still higher concentrations of antigen was prevented by the tendency of BGG to show non-specific coagulation-accelerating effects at concentrations of 500 μg./ml. or higher. Neither this latter effect nor a prozone was seen in experiments with high concentrations of ovalbumin.

Effect of Reaction of Antigens with Natural Antibodies.—The sera of many supposedly normal animals contain antibodies which give cross-precipitin reactions with glycogen and other polyglucoses of similar structure (5). Native dextran has been shown to give such reactions with normal rabbit sera (6), and it was of interest to find that the addition of this polysaccharide antigen to the whole blood of normal rabbits produced an acceleration of clotting like that described above with protein antigen-antibody systems (Table IV). Natural antibodies to the somatic antigens of *E. coli* also exist in normal rabbit sera, as judged by the occurrence of weak but definite precipitin reactions which promptly become stronger following specific immunization (7). It was found

that the addition of *E. coli* lipopolysaccharide to normal rabbit blood also accelerated coagulation (Table IV). In order to determine whether this effect was due to the antigenic or to the "endotoxic" properties of this bacterial antigen, rabbits were made tolerant to it by the daily intravenous injection of increasing doses for 10 days. The animals were then bled and the blood was tested with endotoxin *in vitro*. It was reasoned that if the coagulation-accelerating effect of *E. coli* lipopolysaccharide were due to the endotoxic properties of this material, the blood from the tolerant animals should not

TABLE IV

Effect of Dextran and Endotoxin on Rabbit Blood and Plasma

Clotting system	Antigen added	No. of rabbits	Coagulation time					
			Antigen concentration (*μg./ml.*)					
			0 (NaCl)	0.01	0.1	1.0	10	100
			min.	*min.*	*min.*	*min.*	*min.*	*min.*
Normal blood	Native dextran	5	32.8	29.8	25.2	21.2	20.2	19.0
Normal blood	*E. coli* endotoxin	4	37.3	36.8	35.0	27.5	23.8	19.8
"Tolerant" blood	*E. coli* endotoxin	4	40.6	39.8	36.2	29.2	16.8	12.2
Normal plasma	Native dextran	3	19.3	18.3	16.7	16.7	16.7	—
Normal plasma	*E. coli* endotoxin	6	21.5	—	—	20.5	20.3	20.7

Each tube contained 0.1 ml. antigen and 2.0 ml. whole blood or 0.1 ml. antigen and 2.0 ml. plasma. The antigen concentrations are the final concentrations in the clotting system, and the coagulation times are averages of multiple determinations, each performed with blood or plasma from an individual rabbit. There was no significant effect when dextran or endotoxin was added to plasma, but both of these substances shortened the coagulation time of normal blood and the *E. coli* endotoxin had at least an equal effect on the blood of "tolerant" rabbits.

exhibit this effect. If, on the other hand, the effect were due to the reaction of the *E. coli* antigen with natural antibodies, the blood of tolerant animals might be expected to react even more than that of controls, because of the increased antibody levels known to be present (8). As may be seen in Table IV, the results of this experiment were more in accord with the latter interpretation.

McKay *et al.* (9) have recently reported a coagulation-accelerating effect of other endotoxins on human blood, and it has been found in this laboratory that native dextran produces a similar effect on normal human blood. These observations suggest that the phenomenon under study is not peculiar to the rabbit, and experiments with immunized laboratory animals of various species are under way to determine whether it is of general occurrence.

Data obtained by McKay *et al.* suggest that the coagulation-accelerating effect of endotoxins on human blood might be mediated by platelets, since

the effect of endotoxin on "platelet-poor" plasma was considerably less marked than on "platelet-rich" plasma. The testing of this point was considerably facilitated in our experiments by the tendency of rabbit platelets to agglutinate spontaneously (4), so that centrifugation of whole blood at 0°C. for 15 minutes at 4,000 times gravity yielded preparations of plasma that were virtually platelet-free. As shown in Table IV, the clotting time of such preparations was not appreciably affected by the addition of either native dextran or *E. coli* endotoxin, and it would thus appear that the phenomenon under study is an indirect effect of antigen-antibody interaction, mediated by platelets and perhaps related to the platelet-clumping effect described earlier (2, 4).

DISCUSSION

In view of the extensive research activity in the fields of immunology and blood coagulation, it seems remarkable that the coagulation-accelerating effect of antigen-antibody reactions has not been previously described. The phenomenon seems to be a general one, as it can be demonstrated in systems involving protein and polysaccharide antigens and with natural antibodies as well as those produced by active immunization. It may also have considerable significance, bearing as it may on the pathogenesis of tissue damage produced by *in vivo* antigen-antibody interaction. The phenomenon would furthermore appear to offer a new and sensitive method for the detection of antigen-antibody interaction *in vitro*, and experiments have been undertaken to determine its possible usefulness in the demonstration of antibodies in transplantation immunity, autoallergic disorders, and other systems in which conventional methods of antibody detection have sometimes yielded equivocal results. It will also be of interest to determine whether non-precipitating antigen-antibody systems produce this effect and whether complement is involved, although the testing of this latter point may be difficult in view of the requirement for fresh whole blood in the test system and of the anticoagulant effect of many anticomplementary substances.

It has not yet been possible to devise experiments to determine whether antigen-antibody interaction is capable of actually initiating coagulation or whether the effect is limited to an acceleration of the clotting process, once this has begun, nor has it been determined whether the phenomenon occurs *in vivo*. If the effect is mediated, as it seems to be, by platelets and is related to the platelet-clumping phenomenon (2, 4), then *in vivo* studies should prove useful since the occurrence of intravascular platelet clumping during antigen-antibody reactions is readily demonstrable (10).

If actual initiation of coagulation can be demonstrated, an interesting possibility will, of course, be raised concerning the mechanism of physiological coagulation initiation. Thromboplastinogen, in tissue extracts or platelets, may contain antigenic material which reacts with natural antibody just as

horse liver glycogen reacts with natural antibody in horse blood (5). The normal initiation of coagulation might be a consequence of the interaction of this antigen-antibody system, with generation of thromboplastin and subsequent involvement of the other components of the clotting mechanism. It is difficult to square this hypothesis with the observation that patients with agammaglobulinemia apparently possess normal coagulation mechanisms (11) unless the term "natural antibody" is broadened to include properdin, which is present in normal concentrations in these patients (12).

At present, it seems desirable to identify the mechanism directly affected by antigen-antibody interaction, so that some more suitable indicator system can be developed or utilized. The technique described for determination of coagulation time does not lend itself well to quantitative studies, because of the lack of a sharp end-point and because of the variable prolongation of the coagulation time obtained in control preparations in successive experiments. A better assay method would permit a more critical approach to some of the problems outlined, and experiments to that end are now in progress.

SUMMARY

Each of several antigen-antibody systems studied has been found to affect the coagulation mechanism in the rabbit, causing a marked shortening of the coagulation time *in vitro* of samples of whole blood maintained in siliconized glassware. Addition of specific antigen to the blood of actively immunized animals or addition of antigen-antibody mixtures to the blood of normal animals produced the effect. The coagulation time of plasma was not affected, indicating that the phenomenon may be mediated by an effect on platelets. This effect of antigen-antibody interaction may be involved in the production of tissue damage *in vivo*.

BIBLIOGRAPHY

1. Opie, E. L., Pathogenesis of the specific inflammatory reaction of immunized animals (Arthus phenomenon), *J. Immunol.*, 1924, **9,** 259.
2. Stetson, C. A., Jr., Similarities in the mechanisms determining the Arthus and Shwartzman phenomena, *J. Exp. Med.*, 1951, **94,** 347.
3. Storck, H., Über hämorrhagische Phanomene in der Dermatologic, *Dermatologica*, 1951, **102,** 197.
4. Stetson, C. A., Jr., Studies on the mechanism of the Shwartzman phenomenon, *J. Exp. Med.*, 1951, **93,** 489.
5. Heidelberger, M., Aisenberg, A. C., and Hassid, W. Z., Glycogen, an immunologically specific polysaccharide, *J. Exp. Med.*, 1954, **99,** 343.
6. Neill, J. M., and Abrahams, I., Reactions of dextrans with antiserums of rabbits immunized with *S. typhi*, *Proc. Soc. Exp. Biol. and Med.*, 1951, **78,** 537.
7. Unpublished data.

8. Cluff, L. E., Studies of the effect of bacterial endotoxins on rabbit leukocytes. II. Development of acquired resistance, *J. Exp. Med.*, 1953, **98,** 349.
9. McKay, D. G., Shapiro, S. S., and Shanberge, J. N., Alterations in the blood coagulation system induced by bacterial endotoxins. II. *In vitro*, *J. Exp. Med.*, 1958, **107,** 369.
10. Abell, R. G., and Schenck, H. P., Microscopic observations on the behavior of living blood vessels of the rabbit during the reaction of anaphylaxis, *J. Immunol.*, 1938, **34,** 195.
11. Good, R. A., and Varco, R. L., A clinical and experimental study of agammaglobulinemia, *J.-Lancet*, 1955, **75,** 245.
12. Good, R. A., and Zak, S. J., Disturbances in gamma globulin synthesis as "experiments of nature," *Pediatrics*, 1956, **18,** 109.