ON THE ANTIGENS OF RED BLOOD CORPUSCLES.

THE QUESTION OF LIPOID ANTIGENS.*

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The existence of a parallelism between the phenomena of specificity as displayed by precipitins for animal proteins on the one hand and agglutinins and lysins for animal cells on the other has generally been taken as a matter of course. It has been pointed out in a previous paper[1] that such a view is not well substantiated. Information is accumulating to the effect that essential differences exist between the antigens involved in reactions of the two sorts. There is good reason to believe that the specificity of precipitins is to be explained by supposing a progressive change in the chemical structure of a special protein, *e.g.* serum globulin, hemoglobin, as one proceeds from a given species to less and less closely related ones. This conception is not in harmony with a number of features of the hemagglutinin and lysin reactions, as for example the existence of isoagglutinins and heterogenetic antibodies. Furthermore, differences can readily be shown between the erythrocytes of species so closely related that their proteins are indistinguishable by precipitin reactions. This may be accomplished by the use of immune agglutinins after partial absorption or even by the agglutinins of normal sera.[1,2] Also a cross-test between the normal sera and the corpuscles of such species may be sufficient to demonstrate their serological dissimilarity. For example, the normal serum of the donkey usually agglutinates horse corpuscles. On the whole there is no sufficient reason for the belief that the hemagglutinin reactions of normal sera have a simple

* Twentieth paper on antigens.

See Landsteiner, K., and van der Scheer, J., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 98.

[1] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1924, xl, 91.

[2] Landsteiner, K., and van der Scheer, J., *J. Immunol.*, 1924, ix, 213, 221.

correlation with zoological consanguinity. Since hemagglutinins and lysins differ in many respects from the known antiprotein immune sera we have drawn the conclusion tentatively that in the former case the antigens are not simple proteins.[1]

The foregoing considerations have led us to resume the investigation of the antigens contained in erythrocytes. Such studies touch upon the question of the antigenic activity of lipoids (*cf.* K. Meyer, Much, H. Schmidt, *et al.*).[3]

The idea that non-protein substances are concerned in the process of serum hemolysis is suggested by the inhibition of the lytic action of sera by ether extracts of erythrocytes[4] and the production of hemolysins by injections of such extracts (Bang and Forssman[5]). The latter observation has been confirmed by several authors.[6] Bang and Forssman inferred that the antigens of red cells causing the appearance of hemolysins are lipoids, a view which has been advanced for several other antigens as well. The deduction of Bang and Forssman has not been generally accepted because of two facts. First, the antigenic activity of the extracts prepared by treating blood cells with organic solvents is weak as compared with that of unaltered cells. Second, a great part of the antigenic property is retained in the material after extraction (*cf.* H. Schmidt[3]).

Thiele and Embleton[7] conclude that the removal of part of or all the lipoids does not interfere with the production of hemolysins and hemagglutinins and Balls and Korns[8] hold a similar view. Accord-

[3] A comprehensive bibliography of this subject is to be found in Landsteiner, K., *Jahresb. Ergebn. Immunitätsforsch.*, 1911, vi, 209. Schmidt, H., Moderne Biologie, Nos. 4, 5, 6, Kabitzsch, Leipsic, 1922, p. 38, 1924. *Cf.* Schmidt, H., *Z. Immunitätsforsch., Orig.*, 1921–22, xxxiii, 216.

[4] Landsteiner, K., and von Eisler, M., *Centr. Bakt., 1. Abt., Orig.*, 1905, xxxix, 309.

[5] Bang, J., and Forssman, J., *Centr. Bakt., 1. Abt., Orig.*, 1905, xl, 151. *Beitr. chem. Physiol. u. Path.*, 1906, viii, 238.

[6] Dautwitz and Landsteiner, Landsteiner and Prašek, Takaki, Wang, Bergel, Schmidt. Thiele and Embleton reported negative results.

[7] Thiele, F. H., and Embleton, D., *Z. Immunitätsforsch., Orig.*, 1912–13, xvi, 160.

[8] Balls, A. K., and Korns, J. R., *J. Immunol.*, 1918, iii, 375. *Cf.* Thorsch, G., *Biochem. Z.*, 1913, lv, 266; von Dungern and Coca, *Münch. med. Woch.*, 1907, liv, 2321, Ritchie, J., and Miller, J., *J. Path. and Bact.*, 1912–13, xvii, 429.

ing to Bennett, C. L. A. Schmidt, and Dement[9] a globulin separated from red cells can cause the production of hemolysins and hemagglutinins.

Since the active substances extracted by organic solvents have not been isolated in a condition of approximate chemical purity the question of their chemical nature must still be regarded as open and their designation as lipoids as only provisional.

In our experiments the antigenic action of alcoholic extracts of erythrocytes has been investigated by the method which has proved serviceable in experiments[10] on heterogenetic antigen; *viz.*, the injection of mixtures of extract with proteins (serum).

EXPERIMENTAL.

Production of Hemolytic Sera with Mixtures of Alcoholic Extracts of Erythrocytes with Serum.

The washed sediment of corpuscles of 1 liter of horse blood was extracted with 2½ liters of 95 per cent alcohol for 24 hours at room temperature. The blood coagulum was extracted a second time with 1 liter of alcohol in the same way. The first extract was evaporated almost to dryness, the residue dissolved in the second extract on a steam bath, filtered hot, and the solution evaporated as before. The residue was finally emulsified by adding salt solution very slowly while stirring and was made up with it to a volume of 50 cc.

Rabbits 1 to 7 were injected with this emulsion after a tenfold dilution with salt solution. 1 cc. of the fluid, therefore, corresponded to 2 cc. of blood.

Rabbits 15 to 21 received a like emulsion but containing one-eighth its volume of filtered pig serum.

Rabbits 8 to 14 were injected with pig serum diluted 1:8 with normal saline.

Rabbits 22 to 26 were injected with an emulsion of blood extract five times as concentrated as that used for the first group of animals. Only two of the five individuals thus treated survived to the end of the experiment.

Rabbits 27 to 29 received an emulsion of a blood extract made with hot alcohol in the proportions as given above for the room temperature or cold extract.

The solutions used for immunization were prepared in a quantity sufficient for all the injections and 0.25 per cent of phenol had been added to them prior to storage in the ice box. The injections were made intravenously at about weekly

[9] Bennett, C. B., and Schmidt, C. L. A., *J. Immunol.*, 1919, iv, 29. Schmidt, C. L. A., and Dement, D. E., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 345.

[10] Landsteiner, K., *Biochem. Z.*, 1921, cxix, 294. Landsteiner, K., and Simms, S., *J. Exp. Med.*, 1923, xxxviii, 127.

intervals. Animals were selected for the injections the sera of which did not hemolyze completely in a dilution of 1:25, and showed none or only a weak agglutination in the same dilution.

Rabbits 30 to 32 were injected intravenously with the washed corpuscles of 3 cc. of horse blood, then twice intraperitoneally with 4 cc.

After several extractions with boiling alcohol of horse blood stromata previously treated with 5 per cent salt solution and weak acid, the residual material still possessed antigenic properties, as shown by the fact that it engendered agglutinins weaker than but similar to those obtained with unchanged blood cells. This residue consisted of an insoluble substance markedly resistant to acids and alkalies. It gave protein reactions but also a positive test with orcinol and copper sulfate.[11] The nitrogen content of this product (about 13.5 per cent) increased gradually as further extractions with alcohol were made. The orcinol test still remained positive.

Tests for Hemolysis.—7 days after the last injection the inactivated sera were tested by adding to 0.5 cc. of progressively doubled dilutions 0.5 cc. of guinea pig serum 1:10 and 2 drops of 5 per cent washed blood cells. The diluted guinea pig serum used caused no hemolysis by itself. Readings were made after 1 hour at 37°C. The figures indicate the ultimate dilution of rabbit serum giving complete hemolysis.

Tests for Agglutination.—To 0.5 cc. of the dilution of the inactivated immune sera 1 drop of 2.5 per cent blood was added. Readings were made after 1 hour at room temperature. The titer indicates the highest dilution in which agglutination could be observed microscopically.

It will be seen from Table I that no increase occurred over the normal, or but a slight one, in the lytic and agglutinative activity from injections of the emulsions of blood extract in the quantities used for Rabbits 1 to 7. The titers were distinctly higher after injections of the extract-serum mixture. These sera, which will be referred to as "extract-immune sera," were not very powerful but the lytic action of some of them equaled approximately that of the immune sera of Rabbits 30 to 32 (see Table II) procured in the usual way by the injection of unchanged erythrocytes in relatively smaller amounts. One may conclude, therefore, that in the case under discussion the addition of serum increases markedly the output of antibodies, as in the experiments with heterogenetic haptene.[12] In the latter case the hypothesis was advanced that a loose combination of the specific substance with proteins might be formed, and as such act as antigen. In experiments with ether extracts of horse blood corpuscles the addition

[11] Levene's reaction.

[12] Landsteiner, K., and Simms, S., *J. Exp. Med.*, 1923, xxxviii, 127.

TABLE I.

Material injected.....	Blood extract.							Pig serum.							Blood extract plus pig serum.							Concentrated blood extract.		Blood extract (hot).		
Rabbit No.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	27	28	29
Titer of hemolysis after six injections.	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25	25	<25	200	400	25	25	25	50	25	<25	50	25	<25	<25
Titer of hemolysis after seven injections.....															400	400	25	25	<25	400			100			
Agglutinin titer after six injections.....	<25	25	<25	50	<25	<25	<25	25	<25	<25	<25	<25	50	<25	200	800	50	25	25	50		50	50	50	25	<25

of pig serum also enhanced the production of antibodies but the ether extracts alone had a higher immunizing power than the alcoholic blood extracts alone.

Properties of the Immune Sera and Differences from Those Obtained by the Customary Method.

Ratio between Lysin and Agglutinin Titer.—The extract-immune sera differed in several respects from ordinary anti-erythrocyte sera. The first point which may be considered is that of the relative lytic and agglutinative strengths. As can be seen from Table II the ratio lysin: agglutinin is distinctly higher for the sera from animals injected with extract mixture. Similar results have been reported in a previous paper as concerns immune sera obtained with ether extracts of red cells.[13]

TABLE II.

Material used for injection	Extract mixture.				Extract alone.	Erythrocytes.		
Immune serum No.	15	16	20	40	23	30	31	32
Titer of hemolysis	400	400	400	400	100	800	400	400
" " agglutination	400	600	400	400	100	3,200	3,200	2,400

The titration of some other common anti-horse blood sera confirmed these results.

Species Specificity.—The extract-immune sera when tested against the red cells of twelve species proved to be species-specific like ordinary anti-horse blood serum. There was, however, a difference between the two in behavior towards donkey erythrocytes. The ordinary anti-horse serum contains a considerable fraction which is not absorbed by donkey blood although active for horse blood.[1,2] The antibodies in the extract-immune sera, on the contrary, are entirely absorbed by donkey blood or nearly so. They act, therefore, on a substance which is apparently common to both horse and donkey blood. Accordingly, the hemolytic titer of the anti-extract-sera is the same

[13] Landsteiner, K., and Prašek, E., *Z. Immunitätsforsch., Orig.*, 1912, xiii, 403. Frouin, A., *Compt. rend. Soc. biol.*, 1907, lxii, 153.

for horse and donkey blood, whereas ordinary anti-horse blood serum has as a rule a distinctly higher titer for horse blood.

The following experiment will serve as an example of several yielding similar results (Table III).

The absorption was made by adding to the immune serum diluted 1 : 10 a half volume of washed blood sediment. The tubes were allowed to stand 1 hour at room temperature and overnight in the ice box.

In experiments of this kind the existence of individual differences between horse bloods must be borne in mind.[1,2]

Inhibition of the Hemolysis by Alcoholic Blood Extracts.—A striking difference between the two sorts of immune serum is seen when their behavior towards emulsions of the alcoholic extracts is tested (*cf.* the experiments of Landsteiner and von Eisler, Bang and Forssman, Bergel, and Schmidt). Whereas under the conditions of our experiments the hemolytic action of ordinary anti-horse blood sera was not inhibited by the addition of the extract, such inhibition occurred in the case of the immune sera prepared with extract plus pig serum or extract alone (Table IV).

The alcoholic extract was prepared by treating the washed sediment of 1 part of horse blood with 2.5 parts of 95 per cent alcohol overnight at room temperature, filtering, extracting again with one volume of alcohol, and combining the filtered extracts. After evaporating the alcoholic solution an emulsion was made with saline so that 1 cc. corresponded to 8 cc. of blood.

To 0.5 cc. of the immune serum diluted 1:100 was added 0.1 cc. of different dilutions of the emulsion of extract and 0.5 cc. 1:10 guinea pig serum.

The test mixtures were kept for 1 hour at 37°C., then 2 drops of 10 per cent horse blood was added.

Readings were made after ½ hour at 37°C.

Similar tests with extracts of the blood of several species—including that of the sheep—and with extracts of horse organs yielded no inhibition under the conditions indicated above.

Flocculation of Emulsions of Alcoholic Extracts.—There is a similarity between the extract-immune sera and Forssman's heterogenetic antisera as concerns inhibition by alcoholic extracts. Furthermore, a precipitation can be demonstrated similar to that which has been observed with the heterogenetic antibodies (Sordelli and Pico, Sachs

TABLE III.

	Blood.	Complete hemolysis up to 1:
Extract-immune serum 16	Horse.	400
" " " 16	Donkey.	400
Same absorbed with donkey blood	Horse.	<10
" " " " "	Donkey.	<10

	Blood.	Agglutinin titer.
Extract-immune serum 15	Horse.	400
" " " 15	Donkey.	400
Same absorbed with donkey blood	Horse.	40
" " " " "	Donkey.	<10

	Blood.	Complete hemolysis up to 1:
Immune serum 31 prepared with horse blood	Horse.	400
	Donkey.	50
Same absorbed with donkey blood	Horse.	320
" " " " "	Donkey.	<10

	Blood.	Agglutinin titer.
Immune serum 31 prepared with horse blood	Horse.	6,400
	Donkey.	1,200
Same absorbed with donkey blood	Horse.	5,120
" " " " "	Donkey.	<80

Absorption of immune serum 31 was made with a dilution of 1:10 for hemolysis, 1:80 for agglutination. Immune sera 15 and 16 diluted 1:10.

and Guth). As will appear from Table V, this latter phenomenon is also manifested by ordinary anti-horse blood sera but less regularly and usually with less intensity.

TABLE IV.

0.1 emulsion of alcholic diluted 1:	1:1	1:4	1:16	1:64	1:256	NaCl
Extract-immune-serum 15.	0	0	0	0	D.	C.
" " " 16.	0	0	0	F. tr.	D.	C.
" " " 20.	0	0	0	F. tr.	Str.	C.
" " " 40.	0	0	0	D.	V. str.	C.
Anti-horse blood serum 30.	C.	C.	C.	C.	C.	C.
" " " 31.	C.	C.	C.	C.	C.	C.
" " " 32.	C.	C.	C.	C.	C.	C.

C. = complete hemolysis; V. str., very strong; D., distinct; and F. tr., faint trace.

TABLE V.

Dilution of the immune serum.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	Tubes that cleared up.
Extract-immune serum 15.....	3	3	3	3	2	½	Tr.	—	5
" " " 16.....	3	2	1	1	½	Tr.	—	—	4
" " " 20.....	2	2	2	1	1	½	Tr.	—	5
" " " 40.....	3	2	1	½	½	Tr.	—	—	4
Anti-horse blood serum 30.....	½	Tr.	Tr.	—	—	—	—	—	—
" " " 31.....	—*	—	—	—	—	—	—	—	—
" " " 32.....	2	1	½	Tr.	—	—	—	—	2
" " " 33.....	½	½	Tr.	—	—	—	—	—	2
" " " 34.....	1	½	½	Tr.	Tr.	Tr.	—	—	4
" " " 35.....	½	½	Tr.	—	—	—	—	—	1
Anti-donkey blood serum 36.....	½	Tr.	Tr.	—	—	—	—	—	1
" " " 37.....	3	1	½	½	½	Tr.	—	—	4
" " " 38.....	—	—	—	—	—	—	—	—	—
Anti-human " "	—	—	—	—	—	—	—	—	—
Anti-chicken " "	—	—	—	—	—	—	—	—	—
Anti-guinea pig blood serum.....	—	—	—	—	—	—	—	—	—
Anti-rat " "	—	—	—	—	—	—	—	—	—
Anti-sheep " "	—	—	—	—	—	—	—	—	—

* Small flakes to be seen in the first three tubes. No distinct sediment.

The technique was as follows: The alcoholic extract was prepared as for the inhibition tests and then concentrated to one-half of the original blood volume. To 1 cc. of this fluid was added, drop by drop, 5 cc. of saline, as in the Sachs-Georgi

test; no cholesterol was added. To a series of small tubes (inside diameter about 7 mm.) containing 0.2 cc. of the immune serum in increasing dilutions 0.2 cc. of the emulsion was added. The control tube contained 0.2 cc. saline in place of the diluted serum, it showed no flocculation under the conditions of the experiments; *i.e.*, after keeping the tests for 20 hours at 37°C.

The figures indicate the intensity of the flocculation as shown by the sediment at the bottom of the tubes. The figures in the last column (Table V) give the number of tubes in which the liquid became distinctly clear as compared with the control tubes.

Of nine anti-horse blood sera, only two, and of five anti-donkey sera two gave a completely negative reaction. A distinct though not strong reaction was shown by two heterogenetic sera prepared by injection of horse kidney. These sera had a considerable hemolytic titer for horse blood.

Twenty-two heterologous anti-erythrocyte sera effective against various kinds of blood were tested. Two of them only gave a weak reaction.

DISCUSSION.

In our opinion the results reported are adequate to account for the conflicting views advanced by previous authors. In erythrocytes there evidently exists more than one substance capable of generating lytic and agglutinating antibodies; for after an extraction of active substances with alcohol the stromata still contain a potent antigen. That there exist diverse antigenic factors is also shown by the fact that two characteristic types of immune sera were obtained by using either erythrocytes or extracts for immunization.

The active substances in the extracts have properties very similar to the material extracted from heterogenetic antigen (*cf.* H. Schmidt) and termed by us heterogenetic haptene. And for the same reasons as in the latter case, they are probably not proteins. Like the heterogenetic haptenes the substances are easily soluble in alcohol, at least in their state of impurity, and have a slight immunizing activity which is enhanced by the addition of protein solution (serum). The substances react with the corresponding antibodies in the same way as the heterogenetic haptene. The interaction is shown by the inhibition of hemolysis and by flocculation.

There is a marked difference, however, between the heterogenetic substances and those present in horse blood. The former occur in the organs of many animals, while the latter have not so far been found elsewhere than in the blood of horses, donkeys, and mules and seem, therefore, to be peculiar to the species and the nearest relatives. Presumably substances with like properties will be found in other erythrocytes. They have been observed in other cells. (See Meyer, Much, Schmidt, and others.) The fact that horse hemolysins produced in the customary way by injecting unchanged blood may give flocculation reactions with alcoholic blood extracts can be ascribed to the existence in ordinary hemolysins of a fraction of antibodies corresponding to those of the extract-immune sera.

CONCLUSIONS.

Erythrocytes contain more than one substance responsible for the production of lysins and agglutinins.

By injections of alcoholic extracts of horse erythrocytes mixed with a foreign serum, hemolysins and agglutinins can be more readily obtained than by the same quantities of extract alone. The immune sera reacted on horse and donkey blood but not on the blood of other species.

The antibodies obtained differ from those prepared in the usual way with unchanged red blood corpuscles. The differences involve the ratio lysin : agglutinin, the specificity, the inhibition of hemolysis by alcoholic extracts, and the flocculation of these extracts.

Ordinary anti-horse sera give flocculation reactions with emulsions of alcoholic extracts of corpuscles but to a lesser degree and not so regularly as those produced by means of extracts.

The specific substances present in alcoholic blood extracts are probably not proteins. According to this view, species specificity in animals depends not alone on proteins but also on another group of substances.