POSITIVE DIRECT COOMBS TEST INDUCED BY PHENYLHYDRAZINE [1]

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The Coombs or antiglobulin test was introduced for the detection of antibodies to erythrocytes of a type termed "incomplete antibodies" (1). The indirect Coombs test has gained wide application in the detection of the antibodies to various erythrocytic antigens (2) and in the cross-matching of blood for transfusions (3). The direct Coombs test has become a diagnostic and investigative tool in hemolytic disease of the newborn (4–6), following incompatible blood transfusions (7) and in the acquired hemolytic anemias, idiopathic or secondary types (8–22).

All of these conditions in man when associated with positive direct and indirect Coombs tests, have been interpreted to be the result of immune mechanisms, either of the isoimmune or autoimmune type. In the present communication the production of a positive direct Coombs test in the dog by means of the drug phenylhydrazine is described. The phenomenon is unattended by the immunologic implications of blood incompatibility and is not associated with the usual conditions causing a secondary or symptomatic acquired hemolytic anemia.

MATERIAL AND METHODS

Adult normal mongrel dogs of either sex weighing 5.5 to 11 kg. were used. After the control observations had been completed, a 1 to 2 per cent solution of phenylhydrazine hydrochloride (Merck) in saline was injected intravenously in a single dose of 40 mg. per kg. body weight. The studies were conducted periodically thereafter up to 41 days.

The hemoglobin concentration of the peripheral blood was determined by the alkaline hematin method (23). The hematocrit was determined according to Wintrobe (24) and the reading was corrected for trapped plasma according to the method of Chaplin and Mollison (25). Reticulocytes were estimated routinely by counting 1000 erythrocytes stained with brilliant cresyl blue (26). Heinz bodies were stained with 0.2 per cent methyl violet in 95 per cent ethyl alcohol and the percentage concentration of erythrocytes containing these structures was obtained by counting 1000 erythrocytes (27). The mechanical fragility of erythrocytes was determined by a modification of the method of Shen, Castle, and Fleming (28). One cc. of oxalated blood (5 cc. blood plus 10 mg. of a buffered mixture of potassium oxalate and ammonium oxalate) was placed in a 25 cc. Erlenmeyer flask containing 20 glass beads, each of about 4 mm. diameter. The flask was clamped to a plastic disc 16.5 cm. from the axis and rotated for 90 minutes at 30 rpm. at room temperature. The amount of hemoglobin in the supernatant plasma was related to the total hemoglobin concentration of the blood as per cent hemolysis.

The plasma hemoglobin concentration was determined by the method of Bing and Baker (29). The serum bilirubin concentration was determined by the method of Malloy and Evelyn (30).

The Coombs tests: The canine serum anti-serum was prepared in the rabbit by slight modifications of the directions of Mourant (31): Rabbits weighing 2 to 3 kg. were injected with pooled canine serum obtained by mixing about 5 cc. of serum from each of three to five dogs. A course of intravenous injections was given at 2-to-3-day intervals beginning with a 0.5 cc. dose and following with 5 doses of 1 cc. each. The animal was bled 10 days after the last dose. Pooled erythrocytes from normal dogs were used in absorbing the rabbit serum. The serum was then passed through a Seitz filter and aliquots of 1 cc. were stored in small sterile bottles at $-20°C.$ until used. The results with this serum were compared on nine occasions with the results using a canine antiglobulin serum kindly furnished by Drs. L. E. Young and S. N. Swisher of Rochester, New York. When these two sera were used undiluted with cells giving positive results, the degree of agglutination evoked by the two was identical.

In the direct Coombs test one drop of a 2 per cent suspension of the canine erythrocytes was washed three times in abundant saline at room temperature. After the third wash the test tube was turned up and allowed to drain. To the erythrocytes suspended in the residual saline, a suspension approximating the original 2 per cent concentration, 2 drops of the canine serum anti-serum (Coombs serum) were added. The mixture was allowed to stand for 30 minutes following which it was centrifuged for one minute at 500 to 1000 rpm. After shaking the button of cells into the supernatant the results were evaluated grossly as follows: a solid button

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of agglutinated cells was designated 4+, a few large clumps 3+, multiple scattered smaller clumps 2+, a definitely granular suspension with fine clumps 1+. All of these grossly detectable degrees of agglutination were checked by microscopic inspection which also demonstrated that the clumps of erythrocytes were stable for periods longer than 5 minutes. A smooth or fairly smooth suspension which upon microscopic inspection yielded definite but scattered clumps was designated ±. Rare microscopic clumps in a grossly negative preparation were ignored and the test was called negative. Since the ± designation is questionable, no significance is given to it in this presentation. The microscopic appearances are depicted in Figure 1.

The indirect Coombs test was conducted by incubating erythrocytes from normal dogs as a 2 per cent suspension at 37° C. for 30 minutes in the serum of the dogs receiving phenylhydrazine and then treating the cells as in the direct procedure. A random panel of canine erythrocytes obtained from three normal dogs was used throughout any one experiment.

The titration of the Coombs serum, with minimal modification, was conducted in accordance with the suggestion of Evans and Duane (11). Serial dilutions of the canine serum anti-serum with saline were prepared. To 0.1 cc. of the diluted serum was added 0.05 cc. of a 2 per cent suspension of washed erythrocytes. The mixture stood at room temperature for 30 minutes, then was

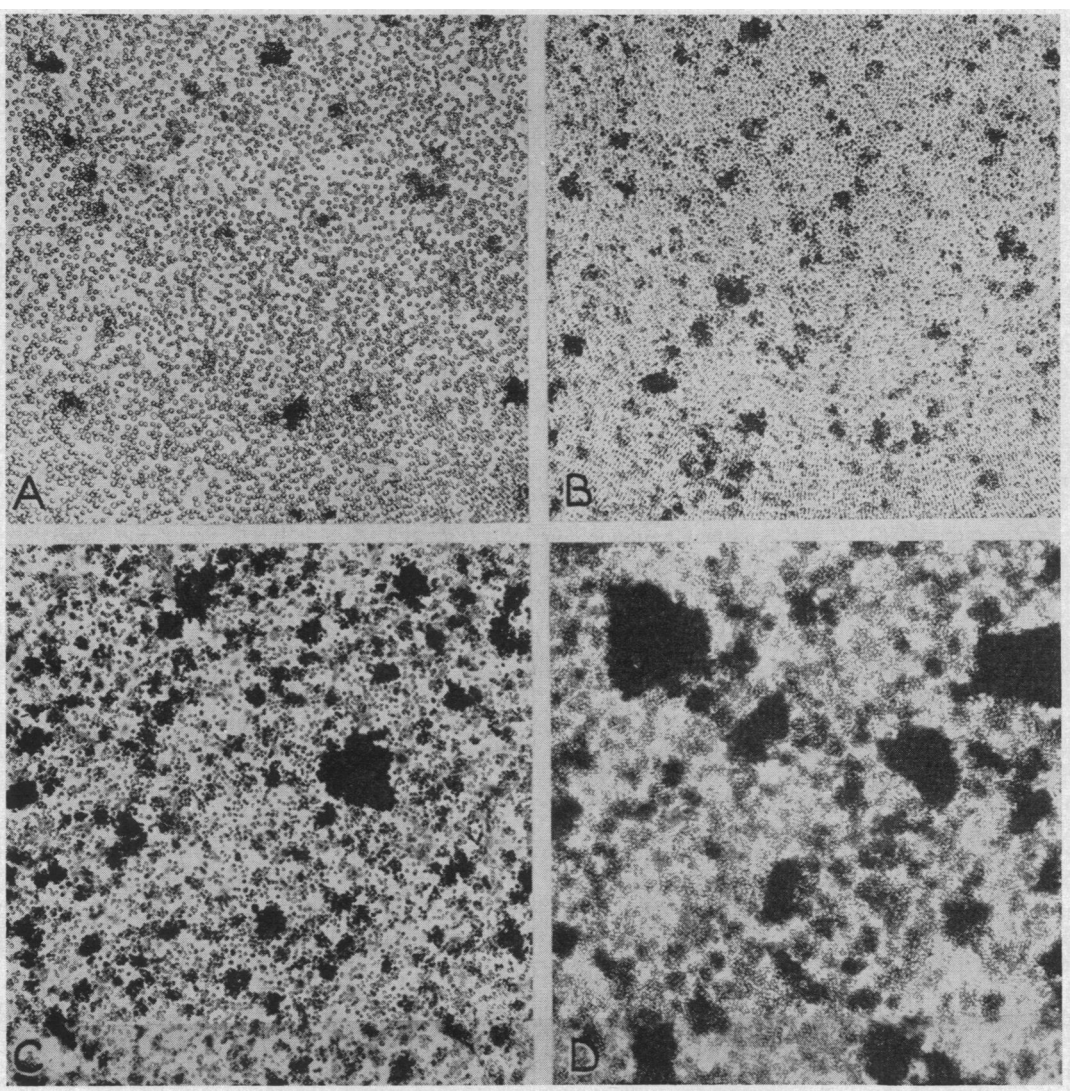

FIG. 1. MICROSCOPIC APPEARANCE OF THE VARIOUS GRADES OF AGGLUTINATION INDUCED BY THE DIRECT COOMBS TEST FOLLOWING PHENYLHYDRAZINE. (A) DEPICTS THE MICROSCOPIC APPEARANCE OF A GROSS ± REACTION; (B) A 1+ REACTION; (C) A 2+ REACTION; AND (D) A 3+ REACTION

centrifuged at 500 to 1000 rpm. for one minute and evaluated for clumping.

The trypsin-treated erythrocytes were prepared by a modification (32) of the description of Morton and Pickle (33). A 2 per cent solution of trypsin (Difco 1:250) in buffered saline (pH 7.3) was stored at −20° C. in small aliquots until used. A 1:10 dilution of this 2 per cent solution in buffered saline was used as a working solution. To 4.5 ml. of a 10 per cent suspension of washed RBC was added 0.5 ml. of the diluted trypsin solution. This mixture was incubated for 10 minutes at 37° C., centrifuged at 2500 rpm. for 10 minutes and the supernatant decanted. The cells were re-suspended to 2 per cent suspension in the buffered saline before using.

The test for "incomplete antibodies" using a colloid medium (34) was performed by the use of a final suspension of erythrocytes in 22 per cent human albumin solution according to the following steps: The 22 per cent solution was made up from a 25 per cent solution of human albumin in buffered diluent (E. R. Squibb and Sons, New York) by adding buffered saline (pH 7.3 to 7.5). To 0.1 cc. of 22 per cent albumin in a 13 × 75 mm. test tube was added 1 drop of a 2 per cent suspension of the test cells which had been washed three times in abundant saline. This was incubated at 37° C. for 30 minutes, centrifuged at 500 to 1000 rpm. for 1 minute and examined macroscopically for clumping. If no gross agglutination was evident the contents of the tube were poured on a slide and examined microscopically.

The eluates from the Coombs positive erythrocytes were prepared by an adaption of the Landsteiner-Miller procedure (35) as follows: 15 to 20 cc. of defibrinated blood were centrifuged, the serum was removed, the erythrocytes were washed four times with abundant saline, a final 50 per cent suspension of erythrocytes in saline was heated to 56° C. with constant agitation for 6 to 8 minutes. The saline was then separated while warm. As a result of this procedure hemolysis was mild to moderate. The ability of such eluates to produce direct agglutination and agglutination by means of the indirect Coombs procedure was tested by means of a panel of erythrocytes obtained from normal dogs. No direct agglutination was noted.

Since phenylhydrazine produces intravascular hemolysis and hemoglobinemia control tests were conducted in an attempt to determine whether hemolyzed canine blood *per se* could cause the erythrocytes obtained from normal dogs to agglutinate under the influence of the canine antiserum produced in the rabbit (canine Coombs serum) and used in the experiments here reported. The normal dogs used in these tests were not used for the injection of phenylhydrazine.

For this purpose the indirect Coombs test was conducted on the serum and hemolyzed erythrocytes obtained from two normal dogs. The serum and hemolyzed erythrocytes were incubated with the erythrocytes obtained from three additional normal dogs and the erythrocytes from the dog contributing the serum and hemolyzed erythrocytes. The incubation and the remainder of the indirect Coombs test was conducted as described above. The hemolyzed erythrocytes were prepared as follows: the erythrocytes from the normal animals used were washed three times with abundant saline, a final suspension of erythrocytes in saline was prepared with the same concentration of erythrocytes as originally present in the blood, this suspension was hemolyzed by repeated freezing and thawing. Altogether there were eight tests against the sera and eight tests against the hemolyzed fractions. In all instances the indirect Coombs test was negative.

The influence of hemolysis *per se* on the Coombs test was appraised also by the injection of 300 cc. of hemolyzed blood, obtained by repeated freezing and thawing, into a previously unused normal dog. The effect of this procedure on the direct Coombs test, Heinz body concentration and mechanical fragility of the recipient's circulating erythrocytes was noted periodically for one week. These tests yielded negative results.

These observations indicated that hemolysis *per se* did not cause a false positive Coombs test with the canine antiserum prepared in this laboratory.

RESULTS

Direct Coombs test

a) Results with the undiluted Coombs serum (Table I). The results from seven dogs followed up to 41 days after the injection of phenylhydrazine are summarized in Table I. The direct Coombs test became positive within two to three days. The gross agglutination varied between 1 and 3 +. The test was observed in these dogs to remain positive for 16 days. It became negative between 16 and 28 days. Dogs Nos. 6 and 7 (Table I) received additional doses of phenylhydrazine after the positive direct Coombs test had disappeared. The cycle of positive direct Coombs test repeated itself in a manner similar to that observed following the first injection.

b) Results with serial dilutions of Coombs serum with saline; the titer of Coombs serum; the prozone phenomenon (Tables II, III, and IV). Serial dilutions of the canine-serum anti-serum (Coombs serum from the rabbit) were prepared with saline. The diluted serum was added to washed 2 per cent suspensions of the canine erythrocytes after the injection of phenylhydrazine. The procedure was conducted periodically with the red cells from dogs Nos. 1, 2, 3, 7, 8, 9, 10, and 11 as shown in Tables II, III, and IV. The dilutions of 1:8 to 1:512 yielded positive results at 3 to 6 days. Thereafter the titer gradually receded except for dog No. 3, which expired and revealed a terminal elevation of the titer.

Absent or weaker agglutinations at lower dilutions followed by stronger agglutinations at higher dilutions were noted in 8 of 32 titrations on the 8 dogs tested. This phenomenon had the characteristics of a prozone (36) on four occasions in the sense of a gradual elevation in the intensity of agglutination as succeeding higher dilutions of the serum were used. In the other four examples the phenomenon was prozone-like in that the change was sudden from no agglutination to a positive result with higher dilutions. This blocking out of the positive test with the undiluted serum possibly explains the negative results noted with dogs Nos. 3, 5, and 7 on days 6, 13, and 5, respectively,

TABLE I

Phenylhydrazine (40 mg. per kg.) was given following the observations on zero day *†

Dog No. Wt. *kg.*	Day	Direct Coombs test	Hb. *gm./100 cc.*	Hematocrit %	Heinz body conc. %	Mech. fragility *% Hem.*	Reticulocytes %
	0	Neg.	11.0	36.4	0	4.4	0.3
	1	Neg.	9.4	31.3	58.0	29.5	0.7
	3	1+	6.1	20.7	82.2	31.1	1.8
	6	1+	2.2	14.0	54.0	25.3	11.4
	10	1+	6.0	24.5	7.6	28.2	7.6
(1)	14	1+	7.3	29.8	0.2	56.7	5.7
	16	1+	7.1	27.5	0.1	43.9	1.4
11	20	±	8.7	29.7	0	45.0	0.7
	24	±	7.6	31.4	0	15.2	0.6
	28	Neg.	8.9	29.4	0	67.3	
	30	±	10.8	34.5	0.1	33.9	0.1
	35	Neg.	9.2	31.2	0	57.4	0.3
	41	Neg.	11.8	32.4	0	17.0	0.5
	0	Neg.	14.0	43.3	0	7.0	0.6
	1	±	10.2	33.3	60.0	13.0	1.8
	3	1+	7.9	24.6	75.7	47.7	4.4
	6	1+	6.6	26.1	41.5	28.7	9.9
(2)	10	±	9.4	30.9	30.0	54.2	2.6
	14	±	9.8	37.2	1.5	58.3	2.9
6.4	17	±	10.6	36.3	0	35.2	1.1
	20	±	12.0	39.2	0	6.2	0.5
	24	Neg.	10.6	37.2	0	40.6	0.3
	28	Neg.	10.8	39.1	0	81.6	
	30	±	12.5	40.4	0.1	24.8	0.1
	35	Neg.	12.8	37.2	0.9	9.2	0.1
	41	Neg.	13.5	43.5	0	40.0	0.4
	0	Neg.	14.4	44.9	0	7.3	0.1
	1	Neg.	10.0	37.2	72.0	12.5	0.4
	3	1+	7.2	23.0	85.6	73.4	1.3
(3)	6	Neg.	4.7	19.7	48.0	50.0	14.6
	10	1+	5.2	20.6	16.1	97.0	3.4
	14	±	5.2	22.8	0.3	21.3	1.9
5.5	17	3+	6.0	23.1	0.2	40.8	2.9
	Expired						
	2	1+	14.0		47.0		
	3	1+	7.9	26.0	60.0		
	6	3+	3.5	16.0	64.0		
	7	3+			52.0		
(4)	12	2+			9.0		
	13	1+			5.8		
11	15	1+	6.4	27.2	4.7		
	16	Neg.			1.6		
	19	Neg.	7.5	29.9	1.4		
	21	Neg.	8.0				
	28	Neg.			0.7		

* The direct Coombs test was obtained by the use of the undiluted canine serum anti-serum (Coombs serum).
† Hb: Hemoglobin concentration.
Heinz body concentration: Concentration Heinz body % of 1,000 RBC.
Mechanical fragility: Mechanical fragility RBC % hemoglobin liberated.

TABLE I—*Continued*

Dog No. Wt. *kg.*	Day	Direct Coombs test	Hb. *gm./100 cc.*	Hematocrit %	Heinz body conc. %	Mech. fragility % *Hem.*	Reticulocytes %
	2	1+	11.5	27.5	54.0		
	3	1+	8.4	23.0	61.0		
	6	2+	5.1	20.0	47.0		
	8	3+			39.0		
(5)	12	1+		30.0	5.0		
	13	Neg.		28.0	3.7		
6.4	15	3+	7.6	27.8	1.8		
	16	3+		30.2	0.7		
	19	1+	8.4	30.4	0.4		
	21	3+	9.1	30.0			
	28	±			0.3		
	44‡	Neg.	13.1	41.0	0	5.9	
	45	±	11.6	40.9	74.0	22.0	
	48	±	8.0	30.2	77.4	42.6	
	51	±	11.2	34.3	31.8	11.8	
(6)	56	±	10.6	33.9	6.7	37.8	
9.0	66	1+	11.0	34.0	0	27.0	
	69	±	10.6	34.1	0	76.4	
	85§	Neg.	10.6	34.1	0	5.6	
	87	±	7.1	36.4	83.6	33.8	
	91	1+	5.8	21.8	57.9	45.1	
	93	1+	7.6	25.5	23.6	10.3	
	98	±	8.4	30.4	2.7	55.5	
	0	Neg.	10.9	35.4	0	5.8	
	1	±	9.6	35.4	29.0	9.0	
	3	1+		37.0	52.0	25.0	
	5	Neg.	9.6	32.0	32.0	14.3	
	7	2+	7.1	26.3	16.0	3.2	
	10	1+	7.1	29.7	1.5	6.5	
	13	1+	10.2	35.6	0.2	8.2	
(7)	18	Neg.	10.2	35.6	0	10.2	
11.4	28	Neg.	11.8	38.3	0	5.4	
	47‡	Neg.	12.3	40.0	0		
	49	1+	7.1		92.2	27.3	
	50	2+	7.6			15.7	
	53	2+	4.4	19.3	56.5	7.8	
	55	2+	6.9	25.4	12.8	4.5	
	60	±	8.1	30.4	6.7	13.8	

‡ Second dose of phenylhydrazine 44 and 47 days after first dose.
§ Third dose phenylhydrazine 41 days after second dose.

as shown in Table I. These negative results with the undiluted serum were preceded and followed by positive results.

c) Potentiation of the positive direct Coombs test with trypsin (Table III). Subjecting the erythrocytes to the action of trypsin prior to the addition of the canine serum anti-serum accentuated the degree of agglutination. The treated cells also carried the test in the titration of the serum one to several tubes beyond that observed without the action of the enzyme as noted in eight comparisons conducted on dogs Nos. 1, 2, 7, and 9. Trypsin alone, under the conditions used, was not able to affect agglutination of the erythrocytes. Eventually, even with trypsin, the test became negative.

d) Neutralization of the Coombs serum from the rabbit with canine serum (Table IV). The Coombs serum was diluted serially with saline for one set of tests. For another set of tests on the same erythrocytes the Coombs serum was diluted serially in the same manner with canine serum prepared by pooling equal parts of serum obtained from four normal dogs. The Coombs serum and normal canine serum were allowed to interact for 30 minutes before use. The erythrocytes tested yielded a positive direct Coombs test following the injection of phenylhydrazine. This procedure was

TABLE II

Data on the Coombs titer obtained by serial dilution of the Coombs serum with saline and its addition to washed erythrocytes following the injection of phenylhydrazine

Dog No.	Day	Serial dilutions of Coombs serum 1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
(1)	3	0	0	0	+	+	+	+	+				
	5	+	+	+	+	+	±	±	±	±	±	0	
	10	+	+	+	+	±	±	±	±	0	0	0	
	14	+	+	+	+	±	±	±	±	0	0	0	
	16	+	±	±	±	±	±	±	0	0	0		
	20	±	±	±	±	±	±	±	±	±	±	0	
	24	±	±	±	±	±	±	0	0	0	0	0	
	30	±	±	±	0	0	0	0	0	0	0	0	0
(2)	6	+	+	+	+	+	0	0	0	+	±	0	0
	14	±	±	±	±	0	0	0	0	0	0	0	
	17	±	±	±	0	0	0	0	0	0	0		
	20	±	±	0	0	0	0	0	0	0	0	0	
	24	0	0	0	0	0	0	0	0	0	0	0	
(3)	3	+	0	0	0	0	0	0	0	+	+	±	0
	6	0	0	0	0	0	±	±	±	±	±	±	0
	10	+	±	±	±	±	±	±	±	+	0	0	0
	14	±	±	±	+	++	+	+	+	±	±	±	0
	17 Expired	+++	+++	+++	+++	+++	++	++	++	±	0	0	0

conducted a total of seven times on the erythrocytes from four dogs (Dogs Nos. 8, 9, 10, and 11). The direct Coombs test was positive with the saline dilutions of the Coombs serum of 1:8 to 1:64. Dilution and incubation of the Coombs serum with the pooled canine serum neutralized the ability of the Coombs serum to cause agglutination of the erythrocytes in six of the seven tests conducted and minimized it on the seventh occasion. This type of neutralization suggests that antibodies in the Coombs serum reacted with corresponding canine proteins on the affected erythrocytes in causing the agglutination.

e) Results with the serum from normal rabbits (Table IV). The washed erythrocytes which yielded a positive direct Coombs test with the

TABLE III

Data on the Coombs titer obtained with untreated erythrocytes and with the same cells after treatment with trypsin

Dog	Day	Coombs method	Serial dilutions of Coombs serum 1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
(1)	24	Regular	±	±	±	±	±	±	0	0	0	0	0	
		Trypsin	++	±	±	±	±	±	±	0	0	0	0	
	30	Regular	±	±	±	0	0	0	0	0	0	0	0	0
		Trypsin	±	±	±	±	0	0	0	0	0	0	0	0
(2)	24	Regular	0	0	0	0	0	0	0	0	0	0	0	
		Trypsin	+	±	0	0	+	+	0	0	0	0	0	
	30	Regular	±	0	0	0	0	0	0	0	0	0	0	0
		Trypsin	±	±	±	0	0	0	0	0	0	0	0	0
(7)	2	Regular	0	±	±	±	±	±	0	0	0	0	0	
		Trypsin		+	+	+	+	±	±	±	0	0	0	
	6	Regular	+	+	+	+	±	±	±	0	0	0	0	0
		Trypsin	+	+	+	+	+	+	±	±	±	0	0	0
(9)	2	Regular	+	+	+	+	+	++	+	±	±	±	0	
		Trypsin	+	+	++	++	++	++	+	±	±	±	±	
	8	Regular	++	++	++	++	+	+	±	±	0	0	0	0
		Trypsin	++	++	++	++	++	++	+	+	+	0	0	0

TABLE IV

The neutralization of the Coombs serum by means of canine serum is demonstrated. Failure of direct Coombs positive erythrocytes to agglutinate in normal rabbit serum, normal canine serum and concentrated albumin is also depicted

Dog No.	Day	Procedure	Serial dilutions of Coombs serum 1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	RBC + Dog serum	RBC + Rab. ser.	RBC + Conc. alb.
8	5	Coombs serum + Saline dilution	1+	1+	1+	1+	+	+	+	0	0	0	0	0	0	0	0
		Coombs serum + Dog serum dil.		±	0	0	0	0	0	0	0	0	0	0			
	7	Coombs serum + Dog serum dil.		±	±	0	0	0	0	0	0	0	0	0			
9	4	Coombs serum + Saline dilution	1+	1+	1+	1+	±	0	0	0	0	0	0	0	0		0
		Coombs serum + Dog serum dil.		2+	±	±	±	±	±	0	0	0	0	0			
10	3	Coombs serum + Saline dilution	1+	1+	1+	±	±	0	0	0	0	0	0	0	0		0
		Coombs serum + Dog serum dil.	0	0	0	0											
	4	Coombs serum + Saline dilution	±	±	±	0	0	0	0	0	0	0	0	0	0	0	0
		Coombs serum + Dog serum dil.	0	0	0	0	0	0	0	0	0	0	0	0			
11	3	Coombs serum + Saline dilution	±	±	1+	2+	1+	2+	1+	±	±	0	0	0	0		0
		Coombs serum + Dog serum dil.				0											
	4	Coombs serum + Saline dilution	1+	1+	2+	2+	1+	1+	±	0	0	0	0		0	0	0
		Coombs serum + Dog serum dil.		0	0	0	0	0	0	0	0	0	0				

saline dilutions of the Coombs serum up to 1:8 to 1:64 failed to agglutinate when placed in the serum from normal rabbits. The same washed erythrocytes also failed to agglutinate in the serum from a normal dog and in a concentrated human albumin solution.

TABLE V

Results of the indirect Coombs test utilizing a panel of 3 or 4 erythrocytes from normal dogs and the serum and eluate from the blood at the time the direct Coombs test was prominently positive

Dog No.	Day	Direct Coombs	Indirect Coombs with	Panel RBC 1	2	3	4
(3)	17	3+	Serum	±	±	1+	
			Eluate	±	±	1+	
(4)	7	3+	Serum	0	0	0	0
			Eluate	0	0	0	±
(5)	7	3+	Serum	0		0	0
			Eluate	±		±	±
(9)*	10	2+	Serum	1+	1+	±	
			Eluate	±	1+	1+	

* Second dose.

The albumin test

It has been demonstrated that "incomplete antibodies" coating erythrocytes can also be detected by agglutination within a colloid medium, such as 22 per cent serum albumin. The albumin test following phenylhydrazine was consistently negative while the direct Coombs test was positive. This observation was made repeatedly with the erythrocytes of eight dogs (Nos. 1, 2, 3, 7, 8, 9, 10, and 11).

Elution of direct Coombs positive erythrocytes (Tables V and VI)

Eluates were prepared by the Landsteiner-Miller procedure from the erythrocytes of six

TABLE VI

The Coombs titer as obtained by serial dilution of the Coombs serum with saline and the addition of this serum to the erythrocytes of three normal dogs after they had been incubated with the serum and erythrocytic eluate both obtained at the time that the direct Coombs test was positive

RBC No.	Serum (S) or Eluate (E)	Serial dilutions of Coombs serum with saline											
		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
1	S	+	+	+	+	±	±	±	±	±	±	0	0
	E	±	±	+	+	+	+	+	+	±	±	±	0
2	S	+	+	±	±	±	±	±	±	±	±	0	0
	E	+	+	+	+	+	+	±	±	±	±	0	0
3	S	±	±	±	±	±	±	±	0	0	0	0	0
	E	+	+	+	+	±	±	±	±	0	0	0	0

dogs (Nos. 1, 2, 3, 4, 5, and 9) at the time the direct Coombs test was positive with the undiluted Coombs serum. The eluate and the serum obtained at the same time were tested by means of the indirect Coombs test against the erythrocytes obtained from three or four normal dogs. In Table V the indirect Coombs test was conducted with undiluted Coombs serum while in Table VI serial dilutions of Coombs serum with saline were used in the indirect Coombs test as applied to the serum and eluate of one dog (No. 9). The eluates of two of the four dogs tested yielded positive indirect Coombs test. The eluates and the corresponding sera gave similar results. These observations indicate that the causative agent of the positive direct Coombs test following phenylhydrazine is elutable but does not elucidate the nature of the eluted material.

The indirect Coombs test (Table VII)

The indirect Coombs test was applied to the serum of the dogs receiving phenylhydrazine utilizing the same panel of erythrocytes from three normal dogs throughout each experiment. It should be noted in Table VII that the indirect Coombs test was negative in all but one case prior to the injection of the drug but that it subsequently became slightly positive in all cases. Although these observations have not been elucidated, they merit some comment.

The development of the positive indirect Coombs test to certain red cells cannot be ascribed to isoimmunization since no blood transfusions were involved. Three alternative explanations for this phenomenon may be considered: 1) That the drug or its hemolytic effects potentiated or unmasked the presence of low titered natural antibodies against certain red cells from other animals; 2) that the material in the serum causing the positive indirect Coombs test was derived from the erythrocytes altered by the drug and yielding a positive direct Coombs test, just as a positive test was obtained by elution; and 3) that the drug altered normal proteins in the plasma, thus making these adsorb onto erythrocytes.

Relation of general hematologic features following phenylhydrazine to the positive direct Coombs test (Table I)

a) Relation to the anemia. It is apparent in Table I that the magnitude of the positive direct

TABLE VII

Data on the indirect Coombs test utilizing the serum following phenylhydrazine and a panel of erythrocytes obtained from three normal dogs

Dog	Day	Indirect Coombs test panel cells		
		1	2	3
(1)	0	0	0	+
	4	±	±	+
	7	±	0	Hem.
	11	+	±	+
	15	+	+	+
	17	0	0	±
	21	+	±	+
	25	±	±	±
(2)	0	0	0	0
	4	±	±	0
	7	0	0	0
	15	±	+	±
	18	+	±	±
	21	±	±	+
	25	+	±	±
(3)	0	0	±	0
	4	+	+	+
	7	0	0	0
	11	+	+	++
	15	+	+	+
	18	±	±	+

Coombs test bore no constant relationship to the degree of anemia induced by the phenylhydrazine. Thus the positive test not only occurred at height of the anemia, as demonstrated by the hemoglobin concentration and the hematocrit reading, but persisted and at times became more prominent during recovery from the anemia.

b) *Relation to Heinz bodies.* In these studies the appearance and disappearance of the Heinz bodies within the erythrocytes, as expressed by the percentage of erythrocytes containing these structures, resembled that described by Cruz (37). Thus the Heinz body concentration reached a maximum in 3 to 6 days, and receded to the base line in 13 to 18 days. There was no constant relationship between the proportion of erythrocytes containing Heinz bodies and the reactivity of the erythrocytes with Coombs serum. The direct Coombs test remained positive after the concentration of Heinz bodies receded to or near extinction. This point was emphasized further in two dogs in which the Heinz body concentration was determined at 1.5, 3, 4.5, and 24 hours following the injection of the drug. The concentration of Heinz bodies at 4.5 hours was 28 per cent for both dogs and at 24 hours the concentrations were 37 and 53 per cent. Yet during the entire interval, the direct Coombs test remained negative, even when serial dilutions of the Coombs serum with saline were used to avoid the prozone phenomenon.

The Heinz body has been interpreted to result from alteration in the structure of the red cell (38, 39). According to Ponder (39) the structure consists mainly of denatured globin. Since it appears to possess a protein component the possibility that the altered protein structure might attract the canine serum anti-serum of the rabbit required consideration. These observations indicate that the Heinz bodies *per se* were not the structures causing the positive direct Coombs test.

c) *Relation to mechanical fragility of erythrocytes.* Following the injection of phenylhydrazine the mechanical fragility of fresh erythrocytes was prominently elevated (Table I) from an average control value of 5.8 per cent hemolysis (4.4 to 7.0 per cent) to values varying between 25 and 97 per cent, usually 57 to 77 per cent. The mechanical fragility reached a maximum at 11 to 15 days in four of five dogs so studied, at which time the direct Coombs test was positive. This degree of mechanical fragility then receded as the direct Coombs test tended to become negative, but in all dogs so studied (Nos. 1, 2, 3, 7, and 9) the fragility reached additional maxima and at times (dogs Nos. 1, 2, 7, and 9) when the direct Coombs test became negative.

d) *Relation to reticulocytes.* The reticulocyte count (dogs 1, 2, and 3) did not become prominently elevated until the anemia reached its maximum. There seemed to be a lag of 3 to 6 days before the upsurge of reticulocytes, despite the prominent stimulus of hemolysis. The reticulocytosis receded at about 15 days. Thus the reticulocytosis was prominent during the interval when the direct Coombs test was positive; however, at times the Coombs test remained positive or increased in intensity (dog No. 3) after the reticulocytosis had receded.

e) *Relation to hemoglobinemia and jaundice.* The positive direct Coombs test was questionable or positive at the height of the hemoglobinemia induced by phenylhydrazine but remained the same or became more positive after the recession of the elevated concentration of plasma hemoglobin. Jaundice was not prominent in these experiments and as a consequence appeared unrelated to the positive direct Coombs test.

Direct Coombs test following addition of phenylhydrazine to blood in the test tube

Phenylhydrazine produces hemolysis and Heinz bodies within the erythrocytes when the drug is added to blood in the test tube, thus demonstrating that the drug injures red cells *in vitro*. This finding suggested the need to test the direct Coombs test following action of the drug in the test tube. Accordingly, to 1 cc. aliquots of oxalated canine blood was added 0.5, 0.25, 0.1, and 0.05 mg. of phenylhydrazine. The first three tubes were allowed to stand at room temperature for 0.25, 1.0, 2.5 to 3.0 hours, respectively, and the last tube was kept in a 37° C. water bath for 18 to 24 hours. At the end of these intervals the erythrocytes were checked for the presence of Heinz bodies and were washed and subjected to the direct Coombs test.

Blood obtained from six normal dogs was so treated. All preparations revealed the presence

of Heinz bodies. In the gross appraisal of the direct Coombs test the erythrocytes in 10 of the 23 tests appeared slightly to moderately clumped (1 + to 2 + results) but upon microscopic inspection the clumps disintegrated after 1 to 3 minutes. The aggregates, which corroborated the gross appearance, appeared to be true clumps and did not resemble coarse rouleaux or so-called red cell "drifts" (4). This transient agglutination suggests a tendency following treatment in the test tube toward the same phenomenon observed after the intravenous injection of the drug. Further observations are required to clarify this possibility. A distinct difference, however, was noted between the agglutination in the direct Coombs test following the injection of phenylhydrazine into dogs, for in these instances the agglutination remained stable for the period observed, which always exceeded 5 minutes and in some special instances exceeded one hour.

DISCUSSION

It is commonly assumed that erythrocytes yielding a positive direct Coombs test are coated with antibodies of the "incomplete type" (1–20). This interpretation, dependent on an antibody response to an antigen, appears definite in certain conditions, such as hemolytic disease of the newborn (4–6) and following certain incompatible blood transfusions (7). In other states the antibody interpretation for a positive direct Coombs test is attractive although not as well founded. Thus, in the acquired hemolytic anemias the positive direct Coombs test has been considered to be a consequence of autoimmune mechanisms (8–17, 40). This concept has gained considerable support by the demonstration of specificities of certain of these "antibodies" in acquired hemolytic anemia (41, 42). Attractive as this hypothesis is the possibility of the production of abnormal globulins capable of attachment to erythrocytes by non-antigenic pathways cannot be ignored (21, 22).

While the literature on the appraisal of the direct Coombs test in man is extensive, few observations on its production in experimental animals have been cited in support of the antibody hypothesis. Noteworthy in this respect are the observations of Young and associates (43–45) on the production of a positive direct and indirect Coombs test in the dog by infusing incompatible blood and in canine hemolytic disease of the newborn in which "incomplete antibodies" were implicated as the causative factor. Chute and Sommers (46) observed a positive indirect Coombs test in parabiotic disease of the rat. The finding was interpreted as resulting from erythrocytic incompatibility causing "incomplete antibodies" in one parabiont which were exchanged through the common circulation and affected the erythrocytes of the partner.

Three important features of the pattern of development of the positive Coombs test in these experiments militate strongly against an antigen-antibody interpretation, and emphasize the weakness of this hypothesis as a general explanation for all instances of positive Coombs test. First, the rapid appearance of the positive Coombs test, within 2 days following injection of the drug, constitutes a strong objection to an antigen-antibody response. Second, the short-lived state of the positive test, as indicated by its disappearance in two to four weeks, is such as to be unusual for an antibody response. Finally, the lack of potentiation of the test by subsequent doses of the drug and the seemingly stereotyped response to subsequent doses do not connote immunologic characteristics.

The drug used, phenylhydrazine, is known to produce direct injury to erythrocytes which is associated not only with hemolysis but also with alterations within the erythrocytes, as demonstrated by the production of methemoglobin and Heinz bodies. The pattern of observations suggests that phenylhydrazine alters the red blood cells in such manner as to change their capacity to react with the canine serum anti-serum of the rabbit. Three theoretical possibilities for these alterations require further experimentation. In one the drug can be considered to alter the surface of the erythrocyte so as to make it act directly as a receptor for the canine antibodies. In another the drug can be considered to alter the surface of the erythrocyte so as to cause it to adsorb non-immune proteins, as normal plasma proteins. Finally, it is conceivable that the drug itself acts as the coupling agent between the surface of the erythrocyte and non-immune proteins, as plasma proteins.

While certain features of this study may be considered to constitute objections to an anti-

body response as the cause for the positive direct Coombs test, the findings of positive indirect Coombs test by the use of the serum and eluates from the erythrocytes have been cited in other circumstances as supporting an immune response. It is difficult to accept such an explanation from the present results, inasmuch as the pattern of development of the positive Coombs test in these experiments has, for reasons previously cited, features which cannot be ascribed to antigen-antibody reactions. An alternative hypothesis would ascribe the positive indirect Coombs test to the same general process of action by the drug which seems responsible for the positive direct Coombs test. Thus, according to this view, the factors causing the positive direct Coombs test might become detached from the erythrocytes, pass into the serum and be reabsorbed by other erythrocytes. It is not clear, as yet, what these factors are. They may be degradation products of injured erythrocytes or altered normal proteins.

These considerations, based on the observations available in the literature and the observations herein recorded, make possible a reappraisal of the mechanisms evoking a positive direct Coombs test. It seems definite that a positive direct Coombs test may result from established antigen-antibody reactions, as in hemolytic disease of the newborn and following certain incompatible blood transfusions. In the acquired hemolytic anemias, idiopathic and certain secondary types, the affected individual's erythrocytes are coated with globulins which resemble "incomplete antibodies." This has led to the interpretation that the globulins are autoimmune, although the presence of abnormal non-immune globulins capable of adsorption to erythrocytes has not been excluded. The present experiments indicate the production of the positive direct Coombs test by the action of a drug, as phenylhydrazine. The findings suggest an action of the drug on the erythrocytes as the cause for this phenomenon.

SUMMARY AND CONCLUSIONS

1. The drug phenylhydrazine has produced a positive direct Coombs test. The agglutination has been stable when the drug was injected intravenously into dogs and transient and unstable when the drug acted on the blood in the test tube.

2. The phenomenon does not appear to result from immunologic mechanisms for the reasons discussed.

3. The positive direct Coombs test appears to result from an alteration of the erythrocyte by phenylhydrazine or some breakdown product of phenylhydrazine.

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