Transfusion of Paraoxon-Treated Homologous Erythrocytes into Rabbits

by F. E. GUTHRIE, J. A. SANTOLUCITO, and E. V. CARUOLO
North Carolina State University, Raleigh, North Carolina

The degree of inhibition of red blood cell (r.b.c.) cholinesterase for diagnosis of poisoning following exposure to phosphate and carbamate insecticides may be a valuable reflection of events occurring in the central nervous system. The first symptoms of poisoning do not usually appear until blood acetyl cholinesterase is reduced to below 40% of normal levels (1). Grob et al. (2) reported symptoms of poisoning in the range of 65-95% inhibition of normal r.b.c. cholinesterase following injections of DFP (diisopropyl phosphorofluoridate) to human subjects. The selective inhibition of certain organophosphates for acetyl- and pseudocholinesterase has been reported a number of times (3) and is most strikingly shown with iso-OMPA (N,N'-diisopropyl phosphorodiamidic anhydride) where the ratio of inhibition of true to pseudocholinesterase is 1:9400 (4). More recently, Main and Braid (5) have reported the selective inhibition of cholinesterases and aliesterases to tri-o-tolyl phosphate.

The role of cholinesterases in mammalian red blood cells has been a matter of speculation. A possible function in membrane transport has been suggested (6, 7, 8), but the data have not established a definitive role.

Bulletin of Environmental Contamination & Toxicology,
Vol. 2, No. 4, 1967, published by Springer-Verlag New York Inc.

It appeared to be of interest to introduce organophosphate-treated r.b.c. using a "total" transfusion technique and to ascertain results of such treatment on surviving animals. Such treatment would ensure that only the r.b.c. cholinesterase was inhibited, whereas the plasma cholinesterase would remain active, allowing assessment of the role of r.b.c. cholinesterase independent of the plasma cholinesterase.

METHODS

Whole blood obtained by either heart puncture or decapitation from New Zealand white rabbits was collected in heparinized (10 units/cc blood) polyethylene containers. The blood was packed and transported in ice and then centrifuged at 2,000 g at 2° C for 10 minutes. The plasma was withdrawn and held at 1° C to be used for final reconstitution of r.b.c.'s just prior to transfusion. The erythrocytes were washed in citrate buffer (2% sodium citrate, 15% glucose, pH adjusted to 7.4 with KH_2PO_4), centrifuged, and the buffer discarded. The washed erythrocytes were then resuspended in citrate buffer containing 2×10^{-5} M paraoxon (diethyl 4-nitrophenyl phosphate) and incubated at room temperature for 20 minutes. They were then washed three successive times with clean citrate buffer. Following the third washing, the erythrocytes were resuspended in the original plasma. Aliquots taken at this time showed the erythrocyte cholinesterase to be 100% inhibited whereas the plasma cholinesterase was not inhibited. Rabbit blood plasma has particularly potent A-esterase

activity (9) making it advantageous in this type of an experiment since phosphate not removed by r.b.c. washing would be rapidly detoxified.

The blood was transferred to blood transfusion bottles and warmed to room temperature. Eight- to ten-week-old rabbits weighing 1.8-2.2 kg. were used for the transfusion experiments. The rabbits were placed in holding cages (10) where the ears were shaved and rubbed lightly with xylene. For phlebotomy, the marginal ear vein of one ear was cut parallel to the vein and vasodilation maintained by placing a test tube filled with warm water (40-50° C) in the ear as far toward the base as possible. Blood was transfused using a 22-gauge needle inserted into the marginal vein of the other ear and attached to a 5 ml syringe equipped with a 3-way stopcock which, in turn, was connected to the blood transfusion bottle. All attachments were made using 0.070" I.D. polyethylene tubing. As 5 ml of blood was removed from one ear of the rabbit, 5 ml of the erythrocyte inhibited blood was slowly added (0.3 to 0.5 ml/10 sec) through the other ear until the desired quantity of blood had been transfused. It required about 30-45 minutes to withdraw and add 100 ml of blood to the unanesthesized rabbits. Although quantities of blood in excess of 100 ml were desired, thrombi in the ear veins did not permit introduction of quantities exceeding those shown in the table. Following transfusion the needle was withdrawn and bleeding stopped by gentle pressure to the ear vein and addition of alum when necessary. The

rabbits were then placed in cages and held for observation. Prior to transfusion, agglutination tests were run with all possible combinations of r.b.c.'s and plasma from donor and recipient rabbits. All agglutination reactions were negative.

Blood samples were taken from recipient rabbits immediately prior to transfusion, at the termination of transfusion, and at various intervals thereafter. Cholinesterase determinations were made by a pH stat method (11), adding 0.5 ml of blood sample to 30 ml of 3 mM acetyl choline chloride, 40 mM Mg Cl at pH 7.1, and 25° C. R.b.c. counts were determined using a Coulter counter, haemoglobin content by the cyanmethhaemoglobin method, and plasma protein content by refractive index. Control rabbits were subjected to identical conditions except the r.b.c. were not inhibited with paraoxon.

RESULTS AND DISCUSSION

The accompanying table summarizes the data. Although the r.b.c. cholinesterase was inhibited to the extent of 50-60% at 18 hours posttransfusion, activity returned to normal levels within 75 to 90 hours. This suggests that the average life span of these infused erythrocytes is short--less than 90 hours. Cholinesterase in neither the plasma used for suspension of the r.b.c. nor the plasma from the treated rabbits was inhibited following transfusion of the treated r.b.c. The number of r.b.c. and the haemoglobin content of the treated rabbits fell to an average of 50% of pretransfusion levels by 18 hours posttransfusion but returned to normal levels by the 3rd to 4th days. However,

TABLE. Cholinesterase activity and r.b.c. number following transfusion of paraoxon-treated r.b.c.

Body wt. kg.	% blood replaced[2]	Paraoxon added	uM acetylcholine chloride hydrolyzed/10 min./0.5 ml enzyme source r.b.c. (above), plasma (below)				R.b.c./mm.[3] x 100 (above) Grams % haemoglobin (below)			
				Hrs. after transfusion				Hrs. after transfusion		
			Before	0.1	16-18	75-90	Before	0.1	16-18	75-90
2.27	46	No	-	-	-	-	-	-	-	-
2.16	61	No	8.2	7.8	8.0	-	-	-	-	-
			4.4	-	4.2	-				
2.16	77	No	9.0	8.0	9.6	-	5,570	5,000	3,650	5,500
			-	-	-	-	10.6	11.0	8.1	10.4
2.27	95[3]	Yes	8.6	3.3	4.0	8.7	-	-	-	-
			3.0	2.8	3.0	-				
1.48	100	Yes	9.4	4.9	3.7	10.6	5,700	4,880	2,720	4,360
			4.4	4.2	4.0	-	11.8	10.9	6.1	10.6
1.25	51	Yes	6.9	5.6	4.9	-	-	-	-	-
			-	-	-	-				
2.16	81	Yes	8.8	4.2	3.7	8.2	6,150	6,070	3,520	5,590
			4.4	4.7	4.2	-	13.6	13.6	9.3	12.9
2.05	90	Yes	8.4	-	3.4	8.0	5,350	4,600	2,600	4,890
			4.6	-	4.3	-	11.8	10.8	5.9	11.0
Composite transfusion blood[1]		Yes	0.0	-	-	-	4,980	-	-	5,080
			3.5				11.1			11.0
Composite transfusion blood[1]		No	9.0	-	-	-	5,200	-	-	5,000
			3.9				10.8			10.8

[1] Held in refrigerator at 2° C.
[2] Calculated blood volume assuming average of 57.3 ml./kg. (16).
[3] 50 ml. blood removed (heart puncture) just prior to transfusion. All other blood volumes normal.

the number of r.b.c. for the control rabbit fell to only 65% of the pretransfusion level and returned to essentially normal levels by the 3rd day. Haemoglobin content decreased proportionately with r.b.c. numbers possibly indicating that the cells were removed from circulation rather than being hemolyzed. Gower and Davidson (12), using glycine C^{14}, showed the normal rabbit r.b.c. life span to be 57 days. Obviously the life span of the transfused r.b.c. herein reported was abnormally short. The reason for this decrease in circulating r.b.c. following transfusion can be explained as due to a combination of factors. Bishop and Surgenon (13) reported 30% decrease at 24 hours of r.b.c. stored in glass containers. Sturma et al. (14) using a double-labeling technique (Cr^{51} and Ashby technique) noted rapid removal of cells following transfusion for partially damaged cells. Return of r.b.c. numbers to normal values within 3-4 days agrees with similar experiments conducted by Cline and Berlin (15) with DFP. The average plasma protein content (limited data on 3 treated rabbits) was found to be 6.2, 5.5, and 6.3 grams per cent immediately before, at 5 minutes, and at 18 hours posttransfusion, respectively.

None of the rabbits showed any symptoms of phosphate poisoning at any time during the experimental period. Heart and respiration rates did not show any change attributable to cholinesterase inhibition. One (1.48 kg.) of the five rabbits transfused with inhibited blood died 48 hours posttransfusion and another (1.25 kg.) 72 hours posttransfusion. However, autopsies

failed to show changes indicative of cholinesterase inhibition. Fourteen days after the transfusion all of the surviving rabbits appeared normal in every respect.

Two rabbits transfused with inhibited blood and three rabbits transfused with normal blood died within 20 minutes of the transfusion, and the cause of death was attributed to lung hemorrhage in each case. This was thought to be a result of too rapid addition of blood during transfusion. These rabbits are not included in the table.

It seems probable that a more successful transfusion (permitting exchange of total blood volume in the order of 90%) could be obtained by cannulation of blood vessels. This would require anesthesia, a treatment not necessary in the procedure reported.

ACKNOWLEDGEMENTS

The assistance of Dr. A. L. Chasson for blood, and A. L. Kaplan for cholinesterase, determinations is gratefully acknowledged. This work was supported in part by PHS ES-00044. Contribution from the Entomology Department, North Carolina Agricultural Experiment Station, Raleigh, North Carolina. Published with the approval of the Director of Research as Paper No. 2321 of the Journal Series.

REFERENCES

1. D. E. HEATH, Organophosphorus Poisons, p. 327 (1961), Pergamon Press, London.
2. D. J. GROB, L. LILLIENTHAL, A. M. HARVEY, and B. F. JONES, Bul. Johns Hopkins Hosp. 81, 217 (1947).
3. R. D. O'BRIEN, Toxic Phosphorus Esters, p. 108 (1960), Academic Press, New York.
4. W. N. ALDRIDGE, Biochem. J. 53, 110 (1953a).
5. A. R. MAIN, and P. E. BRAID, Biochem. J. 84, 255 (1962).
6. M. E. GREIG and W. C. HOLLAND, Arch. Biochem. 23, 370 (1949).
7. W. C. HOLLAND and M.E. GREIG, Am. J. Phys. 162, 610 (1956).
8. I. M. TAYLOR, J. M. WELLER, and A. B. HASTINGS, Am. J. Phys. 168, 658 (1952).
9. W. N. ALDRIDGE, Biochem. J. 53, 117 (1953b).
10. G. W. NANCE and P. SPRADLIN, Turtox News 40, 26 (1962).
11. A. R. MAIN and F. IVERSON, Biochem. J. 100, 525 (1966).
12. D. B. GOWER and W. M. DAVIDSON, Brit. J. Haematol. 9, 132 (1963).
13. C. BISHOP and D. M. SURGENON, The Red Blood Cell, p. 46 (1964), Academic Press, New York.
14. M. M. STURMA, A. DUGAN, and L. S. COLWELL, Blood 19, 115 (1962).
15. M. J. CLINE and N. I. BERLIN, Blood 19, 715 (1962).
16. J. ARMIN, R. T. GRANT, H. PELS, and E. B. REEVE, J. Physiol. 116, 59 (1952).