Oxygen-binding and immunological properties of complexes between dextran and animal haemoglobins

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Complexes of dextran 20000 with haemoglobins of sheep, rabbit, dog, bovine and human origin were prepared through alkylation of haemoglobin by *N*-bromoacetyl-aminoethylamino-dextran. The yields were uniformly high. Complex-formation in each case was accompanied by the disappearance of reactive thiol groups on the haemoglobin, and by an increase in the affinity of the haemoglobin for oxygen. The immunological properties of dog, rabbit and sheep dextran–haemoglobin were investigated in both homologous and heterologous species. The complexes were found to be non-immunogenic in the homologous species. In heterologous species the anti-haemoglobin response induced by each complex was generally of a similar level to that induced by the haemoglobin alone.

Complexes containing haemoglobin (hereafter termed simply Hb) covalently linked to a polymeric carrier such as dextran appear to have considerable potential for blood substitution. By combining the important properties of maintaining volume of the circulation and of transporting oxygen from the lungs to tissues, these materials possess the major criteria required in a clinical blood substitute (Tam *et al.*, 1976; Chang & Wong, 1977; Blumenstein *et al.*, 1978). In addition, the need for blood-typing before infusion would also be avoided.

The results of a previous study suggest that dextran–haemoglobin might be an adequate blood substitute for dogs (Tam *et al.*, 1978), but its exact capability to deliver oxygen under various physiological conditions, the relative efficacy of different haemoglobins and the possible immunogenicity of the molecule are important questions that remain to be investigated. In an attempt to throw light on the two latter questions, complexes were prepared from the haemoglobins of four different animal species and their oxygen-binding capacities were determined *in vitro*. The immunogenicity and antigenicity of three such preparations against both homologous and heterologous hosts also have been examined.

Experimental

Coupling of haemoglobin to dextran

Human haemoglobin was prepared from outdated blood, and sheep, rabbit, dog and bovine haemo-

globins from freshly collected blood as described previously (Chang & Wong, 1977). Dextran of 20000 average mol.wt. from Fisons Ltd., Pharmaceutical Division, Loughborough, Leicestershire, U.K., was converted into the bromoacetyl-aminoethylamino derivative and coupled to each type of haemoglobin at a dextran/haemoglobin weight ratio of 1:3 for 72 h at 4°C. The percentage of Hb coupled into dextran–haemoglobin (hereafter called Dx–Hb) complex was determined by chromatography on a column (2.5 cm × 36 cm) of Sephadex G-150 equilibrated with 0.05 M-sodium phosphate buffer pH 7.5. Flow rate was adjusted to 0.7 ml/min, and 0.2 ml of the sample was loaded on to the column. Fractions of 3 ml were collected with a fraction collector, and the absorbance of the Hb fractions was measured at 576 nm with a Hitachi 139 spectrophotometer.

Chemical properties of haemoglobin

Reactive thiol groups in Hb were determined spectrophotometrically as described by Ampulski *et al.* (1969). The concentration of Hb or Dx–Hb tetramer was adjusted to 0.01 mM in 0.01 M-phosphate buffer, pH 7.0, as measured by the method of Drabkin & Austin (1935). The 4,4'-dipyridine disulphide was supplied by Aldrich as Aldrithiol-4. A typical reaction mixture contained 3 ml of 0.01 mM-Hb and 0.5 ml of 1 mM-4,4'-dipyridine disulphide. The concentration of Hb was measured at 307 nm. The number of reactive thiol groups per

haemoglobin tetramer was calculated from the increase in A_{324} :

$$\text{SH/Hb} = 3.2 (AA_{324}/A_{307})$$

Oxygen-dissociation curves for the various Hb and Dx-Hb samples were determined by the method of Benesch *et al.* (1965) at 25°C in 0.05M-sodium phosphate (pH 7.4)/0.1M-NaCl.

Immunology

(i) *Animals.* Male beagle dogs were obtained from Fisons' breeding colony. They weighed 11–11.5 kg at the start of the experiment. Female New Zealand White rabbits were obtained from L. Moore, Bradford, West Yorkshire, U.K. They weighed 2.0–2.5 kg at the start of the experiment. Sheep of both sexes were purchased at market in Leicestershire and were approx. 9 months of age when used.

(ii) *Antibody assays.* Anti-Hb was measured by two methods. A modified Farr assay based on the method of Garver (1975) was used to measure antigen-binding activity of serum with ^{125}I -labelled Hb. Briefly, 100 μl of serum, diluted 1:10 in 0.1M-sodium borate buffer, pH 8.3, was mixed with an equal volume of ^{125}I -labelled Hb containing 10 μg of protein. After incubation at room temperature for 1 h and overnight at 4°C, bound radioactivity was precipitated by addition of 200 μl of 80%-satd. $(\text{NH}_4)_2\text{SO}_4$. The precipitate was spun down in a refrigerated centrifuge (800 g for 30 min), washed in 0.5 ml of 40%-satd. $(\text{NH}_4)_2\text{SO}_4$ and the radioactivity counted in a gamma counter. From the mean radioactivity (c.p.m.) in duplicate tubes, the percentage of added antigen bound by a known volume of serum was calculated. The specificity of the system was confirmed in preliminary experiments by competitive inhibition with unlabelled Hb.

For passive haemagglutination assays, sera were inactivated at 56°C for 30 min and dilutions made in phosphate-buffered saline (Haemagglutination Buffer, Difco), pH 7.3, in microtitre plates. To each 50 μl of diluted serum an equal volume of 1.5% suspension of tannic acid-treated erythrocytes coated with Hb was added, the mixture shaken and incubated at 37°C for 2 h. Control wells contained 'tanned' erythrocytes without antigen. Anti-dextran antibodies were detected by means of passive haemagglutination (as above) by using erythrocytes labelled with *O*-stearyl-dextran (Hammerling & Westphal, 1967) supplied by Dr. C. Moreno of Wellcome Research Laboratories, Beckenham, Kent, U.K.

(iii) *Injection schedules.* To obtain information on whether the process of coupling Hb to dextran resulted in the formation of an antigenic moiety, the immune response to three Dx-Hb preparations was investigated in the three species (dog, rabbit and sheep) from which the haemoglobins were derived.

The complexes were also administered to heterologous species in order to raise antisera as positive controls and for studies of specificity. To maximize the chances of obtaining responses in the homologous species, repeated small injections of complexes were used rather than few injections of a larger volume. The complexes were also emulsified in Freund's complete adjuvant and injected intramuscularly in some instances. In each animal, blood samples were taken before the first injection and at 2, 4 and 6 weeks thereafter. Serum was separated and stored at -20°C until assayed. All sera were subsequently monitored by the modified Farr assay and by passive haemagglutination to detect and quantify any antibody induced.

Results

Coupling of haemoglobins to dextran

The method for coupling Hb to bromoacetyl-aminoethylamino-dextran was developed originally for human Hb. When the method was tested with sheep, rabbit, dog and bovine haemoglobins, it gave results comparable with those obtained with human Hb. Fig. 1 and Table 1 show the formation of a high yield (>90%) of Dx-Hb with each of the different Hb preparations. Measurements of reactive thiol groups on the different Hb preparations indicated that, without exception, a marked decrease in these

Fig. 1. Coupling of haemoglobin from different species to dextran

○, Elution of uncoupled Hb; ●, elution of the Dx-Hb complex from a Sephadex G-150 column. (a) Human; (b) rabbit.

Table 1. *Synthesis and chemical properties of complexes between dextran and haemoglobins from different species*

Species ...	Human	Dog	Rabbit	Bovine	Sheep
Hb coupled to dextran (%)*	93	95	96	90	95
p_{50} of Hb (mmHg†)	7	7	7	11	18
p_{50} of Dx-Hb (mmHg)	2.8	2.2	2.6	5.0	5.3
$(p_{50} \text{ of Hb})/(p_{50} \text{ of Dx-Hb})$	2.5	3.2	2.7	2.2	3.4
h for Hb	2.7	3.0	2.9	3.0	2.7
h for Dx-Hb	1.5	1.6	1.5	1.5	1.5
Reactive SH/Hb (tetramer)	2	2	2	2	2
Reactive SH/Dx-Hb (tetramer)	0.25	0.15	0.10	0.05	0.14
Reactive SH remaining in Hb after coupling to dextran (%)	12.5	7.5	5.0	2.5	7.0

* Estimated from chromatograms such as shown in Fig. 1 with the aid of a Du Pont 310 curve resolver.

† 1 mmHg $\approx$ 133.33 Pa (SI units).

Table 2. *Immune responses of dogs and rabbits injected with dextran-(canine Hb) complex or canine Hb*

Preinoculation haemagglutination titres: (a) mean value for group < 1; (b) all values are zero; (c) mean value for group = 3.5. Abbreviations used: H.A., haemagglutination; FCA, Freund's complete adjuvant; nd, not determined.

Species	Treatment	Animal no.	Maximum antibody response to each antigen*		
			Haemoglobin		Dextran
			Farr assay†	H.A. titre‡	H.A. titre‡
Dog	6 $\times$ 1.0 ml of dextran-(canine Hb) intravenously	80	1	3 ^(a)	0 ^(a)
		81	0	1	0
Rabbit	2 $\times$ 1.0 ml of dextran-(canine Hb) in FCA	1	nd	12 ^(b)	4 ^(c)
		2	nd	12	4
	2 $\times$ 10 mg of canine Hb in FCA	4	nd	12	0
		5	nd	10	3

* Maximum titres given are highest values reached after subtraction of preinjection values.

† Percentage antigen bound by 10 μ l of serum.

‡ $\log_2$ [Highest serum dilution giving haemagglutination (H.A.) in V-well microtitre plates].

groups resulted from the reaction of each type of Hb with the bromoacetylaminomethylamino-dextran (Table 1).

Oxygen binding by dextran-haemoglobin

The oxygen-dissociation curves for the different species of Hb both before and after coupling to dextran are shown in Fig. 2. Although p_{50} , the half-saturating oxygen partial pressure, varied with the species of Hb, coupling to dextran in each instance brought about a decrease in p_{50} (Table 1), reflecting a tighter binding of oxygen by Hb when the latter is coupled to the dextran carrier. This decrease was accompanied by a diminution in the value of h , the maximal slope of the Hill plot for oxygen binding (Table 1), which is an index of the extent of positive homotropic co-operation between

the subunits within the haemoglobin tetramer (see Wong, 1975).

Immunogenicity and antigenicity of Dx-Hb complexes

(i) *Complex containing canine Hb* (Table 2). Two dogs were each given 1.0 ml of Dx-(canine Hb) complex intravenously once per week for 4 weeks and 2.0 ml for a further 2 weeks. When the sera were tested by means of the Farr assay, no change in binding activity for ^{125}I -labelled Hb was observed relative to preinjection samples, whereas by passive haemagglutination, a very low titre occurred in one animal. This was only transitory however, being present at 2 but not at 6 weeks. All the sera were negative for anti-dextran. Conversely, four rabbits immunized with Dx-(canine Hb) complex or uncoupled canine Hb in Freund's complete adjuvant

Fig. 2. Oxygen-dissociation curves for uncoupled Hb (O) and Dx-Hb complex (●) for different species of haemoglobin (a) Human; (b) bovine; (c) rabbit; (d) dog; (e) sheep. 1 mmHg $\approx$ 133.3 Pa (SI units).

Table 3. Immune responses of rabbits and sheep injected with dextran-(rabbit Hb) complex or rabbit Hb. Pre-inoculation haemagglutination titres: (a) all values are zero; (b) mean value for group < 1; (c) mean value for group = 5. Abbreviation used: i.v., intravenously; other abbreviations are defined in Table 2.

Species	Treatment	Animal no.	Maximum antibody response to each antigen		
			Haemoglobin		Dextran
			Farr assay	H.A. titre	H.A. titre
Rabbit	6 $\times$ 4.0 ml of dextran-(rabbit Hb) i.v.	7	3	0 ^(a)	0 ^(b)
		8	<1	0	0
	6 $\times$ 2.0 ml of dextran-(rabbit Hb) i.v.	9	0	0	0
		10	2	1	0
	6 $\times$ 1.0 ml of dextran-(rabbit Hb) i.v.	11	0	0	0
	2 $\times$ 1.0 ml of dextran-(rabbit Hb) in FCA	13	1	0	0
Sheep	2 $\times$ 0.5 ml of dextran-(rabbit Hb) in FCA	14	0	0	0
		1	51	10 ^(b)	6 ^(c)
	2 $\times$ 0.5 ml of rabbit Hb in FCA	3	58	11	1

produced large amounts of anti-(canine Hb) antibody. Those that received the complex also showed a response to dextran, with a titre equivalent to 1/16, whereas those receiving uncoupled Hb gave only 0 and 1/8 respectively. Interestingly, the maximum anti-(canine Hb) titres attained in the rabbits injected with Dx-(canine Hb) were similar to those in animals given uncoupled canine Hb.

(ii) *Complex containing rabbit Hb* (Table 3). Five rabbits were each given a weekly dose of 1.0, 2.0 or

4.0 ml of Dx-(rabbit Hb) intravenously for a total of 6 weeks. A further two rabbits were given two intramuscular injections of the same preparation, emulsified in Freund's complete adjuvant, 14 days apart, followed 2 weeks later by intradermal injection of Dx-Hb solution (divided between five sites) on the flanks. Analysis of the sera for antibodies to rabbit Hb gave negative results throughout the experiment by both assay methods. All the rabbits were similarly negative for anti-dextran antibody.

Table 4. *Immune responses of sheep and rabbits injected with dextran-(sheep Hb) complex or sheep Hb*
Pre-inoculation titres: ^(a) mean value for group < 1; ^(b) all values are zero; ^(c) mean value for group = 4.6; ^(d) mean value for group = 4.3. For abbreviations, see Tables 2 and 3.

Species	Treatment	Animal no.	Maximum antibody response to each antigen	
			Haemoglobin	Dextran
			Farr assay	H.A. titre
Sheep	6 × 2.0 ml of dextran-(sheep Hb) i.v.	4	<1	0 ^(a)
	6 × 7.0 ml of dextran-(sheep Hb) i.v.	5	0	0
	2 × 1.0 ml of dextran-(sheep Hb) in FCA	7	<1	1.5
	2 × 1.0 ml of sheep Hb in FCA	9	14	3
Rabbit	2 × 10 mg of dextran-(sheep Hb) in FCA	15	4	3 ^(d)
	2 × 10 mg of dextran-(sheep Hb) in FCA	16	29	1
	2 × 10 mg of sheep Hb in FCA	18	24	6

Two sheep immunized with Dx-(rabbit Hb) and uncoupled rabbit Hb respectively in Freund's complete adjuvant produced high titres of anti-(rabbit Hb) antibody and the one that received the Dx-Hb complex also gave a moderate response to dextran. The maximal anti-(rabbit Hb) titres attained in the sheep were again similar whether immunization was with rabbit Hb or Dx-(rabbit Hb).

(iii) *Complex containing sheep Hb* (Table 4). Two sheep were given 2.0 and 7.0 ml respectively of Dx-(sheep Hb) intravenously once per week for a total of 6 weeks. A third animal received two injections of 1.0 ml of Dx-(sheep Hb) in Freund's complete adjuvant 2 weeks apart, followed by multiple intradermal injections (5 × 1.0 ml) of dextran-Hb solution on the back. A fourth animal received uncoupled sheep Hb in Freund's complete adjuvant in the same way. Analysis of sequential serum samples for anti-(sheep Hb) by means of the Farr assay showed no significant increase in antigen-binding activity in any of the animals injected with Dx-(sheep Hb), but a slight increase occurred in the sheep that received only uncoupled sheep Hb in adjuvant. The presence of anti-(sheep Hb) in this animal was confirmed by passive haemagglutination. None of the other sera showed any significant differences from normal preinjection sera.

Serum from all the sheep agglutinated dextran-labelled erythrocytes before injection (at titres of 1/16–1/64) and in the two animals given intravenous injections, the amount of anti-dextran did not alter during the course of the experiment. However, in the sheep injected with Dx-(sheep Hb) and sheep Hb in adjuvant, slight, but transitory, increases were observed. (In view of the pre-existing titre and the observation that the increase was not continued beyond 2–3 weeks, this finding is of doubtful

Table 5. *Positions of cysteine residues in α - and β -chains of different species of haemoglobins (from Dayhoff, 1972)*

Species	Residue no.	
	α -Chain	β -Chain
Human	104	93, 112
Sheep	111	92
Rabbit	104	93
Dog	104, 111	93, 112
Bovine	None	92

significance. The possibility exists that there was a non-specific increase in immunoglobulins induced by the Freund's adjuvant.)

Two of three rabbits immunized with dextran-(sheep Hb) or uncoupled sheep Hb in Freund's complete adjuvant gave significant antibody responses to sheep Hb, but one rabbit given the complex failed to produce anti-(sheep Hb) antibody. There was a minimal response to dextran in two animals that received Dx-(sheep Hb), but an increased anti-dextran titre was also recorded in rabbit no. 18, which received only uncoupled sheep Hb in Freund's complete adjuvant.

Discussion

In the present study the method used for coupling human Hb to bromoacetylaminomethylamino-dextran was found to be equally suited for the high-yield synthesis of a complex between dextran and haemoglobin of sheep, rabbit, dog or bovine origin. Although this result could not have been predicted with certainty, its structural basis becomes apparent from a comparison of the cysteine residues occurring in the various species of haemoglobins (Table 5). It

is known that of the six cysteine residues in the human Hb tetramer, only the two β -93 residues are reactive with thiol-specific reagents under non-denaturing conditions. The two α -104 and the two β -112 residues are buried and not available for reaction. Since there is no cysteine at all in the α -chain of bovine Hb, and no β -112 cysteine in sheep, rabbit or bovine Hb, it is likely that the reactive thiol groups responsible for the formation of the Dx-Hb complex are also the β -93 residues in the case of rabbit and dog Hb, and the immediately neighbouring β -92 residues in the case of sheep and bovine Hb. Table 1 shows the loss of these reactive thiol groups in the process of coupling to dextran.

When human Hb is coupled to dextran, its affinity for oxygen is increased. This behaviour is evidently common to sheep, rabbit, dog and bovine Hb. As Fig. 2 and Table 1 show, the oxygen-dissociation curve in each instance was shifted to the left by coupling to dextran. Although sheep and bovine Hb have a distinctly higher p_{50} than human, rabbit and dog Hb, all five species of haemoglobins underwent a similar 2.2–3.4-fold decrease in the p_{50} on coupling to dextran. These observations suggest that the juxtaposition of the reactive thiol groups and the haem sites is fundamentally the same within the five species of haemoglobins, so that alkylation of the thiol groups produces a similar perturbation on the oxygen-binding function of the haem groups.

With respect to the immunological properties of Dx-Hb, the present investigation shows that, with sheep and rabbits, intravenous administration of homologous dextran-Hb does not induce an antibody response towards either the protein or carbohydrate component of the complex. In dogs there was again no response to dextran but a minimal response to Hb was seen. The significance of this latter response is uncertain, however, as it occurred in only one animal and was transient in nature. In practical terms the amounts of dextran-Hb employed in these experiments were very small relative to transfusion volumes. However, even the smallest dose employed (1.0 ml) contained 60 mg of Hb and 20 mg of dextran, which constitutes a considerable antigenic stimulus (e.g. 6.0 mg/kg in the dog), and it does not necessarily follow that a larger injection volume would induce an immune response. The opposite may even occur as, for example, when large doses of certain antigens induce a state of tolerance rather than immunity (Mitchison, 1968). In rabbits and sheep, negative results were found with both high and low doses of the Dx-Hb complex (maximum equivalent to 1 g/kg in rabbits).

Each of the dextran-Hb complexes tested was found to be immunogenic in heterologous species and in most cases the sera contained antibodies specific for both the Hb and dextran moieties. This suggests that Hb acts as a carrier for the haptenic

antigen, dextran-20000, since linear dextran of mol.wt. less than 100000 is itself non-immunogenic (Kabat & Bezer, 1958). The similarity between anti-Hb titres in animals immunized with Dx-Hb or with uncoupled Hb (e.g. rabbits injected with canine Hb) suggests that complexing to dextran makes little or no difference to the immunogenicity of heterologous Hb, although in rabbits injected with sheep Hb, a decrease in immunogenic potency seems more likely. The number of animals used is too small to establish statistical significance in this regard, however, and further quantitative studies are required.

In conclusion, it has been shown in the present paper that dextran can be coupled with Hb of various species to give essentially similar complexes having well-defined oxygen-binding characteristics. These complexes are uniformly non-immunogenic in homologous species, and their overall antigenic properties in heterologous species are unchanged. The potential value of dextran-haemoglobins as practical blood substitutes is thereby enhanced considerably.

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