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STUDIES ON SPECIES CROSS-REACTIVITY OF HEMOPEXIN BY USE OF MONOCLONAL AND POLYCLONAL ANTIBODIES

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SUMMARY: The extent of immunological cross-reactivity between hemopexins of four species (rat, human, rabbit and chicken) was assessed with four affinity purified polyclonal antibodies and three monoclonal antibodies using RIA, Western blotting and rocket immunoelectrophoresis. Neither the two monoclonal antibodies to rabbit hemopexin (Rb3D11 and Rb3H9), the monoclonal antibody (R4B3) to rat hemopexin nor any of the polyclonal antibodies showed shared antigenic determinants between avian and mammalian hemopexins as judged by RIA or rocket immunoelectrophoresis. Western blotting with polyclonal antibodies revealed some reactivity raising the possibility of a few shared, though distantly related, epitopes. Polyclonal antibodies, raised to the mammalian hemopexins cross-reacted to variable extents with the respective antigens by RIA, results paralleled by data obtained by Western blotting. Anti-rat monoclonal antibodies reacted only with rat hemopexin in Western blots and minimally with rabbit hemopexin in RIA. The anti-rabbit monoclonal antibodies recognized two distinct epitopes one of which is shared with human hemopexin and presumably highly conserved.

Hemopexin (Hx) is a plasma protein that binds porphyrins and metalloporphyrins in an equimolar ratio (1-4) and functions in the transport of systemic heme which it binds with high affinity (5). Heme derived principally from methemoglobin, is transported to receptors on the cell surface of hepatic parenchymal cells (6), taken up by an as yet undefined mechanism, catabolized to bilirubin and the released iron is stored (7). Thus, Hx is an important protein in the conservation of heme iron. It is likely that the heme binding site, as well as the portion of the molecule

ABBREVIATIONS: Hx, hemopexin; McAb, monoclonal antibody; PBS/Tw, phosphate-buffered saline/Tween 20; PcAb, polyclonal antibody; SDS-PAG, sodium dodecyl sulfate-polyacrylamide gel.

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involved in receptor recognition, is phylogenetically preserved. Consequently, some structural resemblance between the Hxs of various species should exist and result in interspecies immunocross-reactivity.

We report here on initial comparative immunological studies using highly purified preparations of three mammalian Hxs and one avian Hx (8) to test for reactivity with polyclonal antibodies (PcAbs) raised against human, rat, rabbit and chicken Hxs, and monoclonal antibodies (McAbs) recognizing rat and rabbit Hx.

Materials and Methods

Rat, human, chicken and rabbit Hxs were purified from plasma (8), based on the method of Tsutsui and Mueller (9). PcAbs were raised in rabbits (against rat, human and chicken Hxs) or in a goat (against rabbit Hx) and purified by affinity chromatography on CNBr-activated Sepharose 4B (Sigma) to which the respective Hxs had been covalently coupled (10). McAbs were developed as to be described (unpublished data) Two McAbs to rabbit Hx (Rb3D11 and Rb3H9) and one to rat Hx (R4B3) were utilized. Rb3D11 and Rb3H9 were purified by Protein A Sepharose Cl-4B (Sigma) (11), and R4B3 was purified using rat Hx coupled to CNBr-activated Sepharose 4B. Antibody concentrations were measured both spectrophotometrically at 280 nm $E^{1\%}_{cm} = 14$, and by the method of Lowry et al. (12). The purity of the preparations was determined by $NaDodSo_4$-PAGE using a 7.5-15% acrylamide gradient gel (13).

RIA was performed essentially as described (14). Briefly, the purified antigens were coated onto 96-well PVC plates at 1 ug/100 ul/well in 0.1 M Na_2CO_3 pH 9.8, by overnight incubation at 4°C. A milk solution (5% non-fat dried milk (w/v), 10 mM Tris, 0.15 M NaCl, 0.1% Nonidet P40, 0.02% sodium azide) (15), was then employed to minimize nonspecific binding. Affinity purified anti-Hx PcAbs or McAbs were allowed to react for 2 h prior to washing with phosphate-buffered saline/Tween 20 (PBS/Tw20). The subsequent detection of bound primary antibody was accomplished using ^{125}I-labeled second antibodies as described below for Western blots. After thorough washing, individual wells were excised and counted in a LKB 1280 UltroGamma counter. Background radioactivity was determined using either normal rabbit or goat IgG or unrelated McAbs.

For rocket immunoelectrophoresis (16), 5% (w.v) PEG 4000 and 0.5 mM calcium lactate were added to enhance precipitation (17). In order to optimize the ratios of antigen to antibody concentrations for precipitation, several antigen and antibody dilutions were tested.

Western blots (18) were performed as follows: the denatured samples were electrophoresed on 7.5-15% gradient $NaDodSO_4$-PAGE and subsequently equilibrated for 15 min in blotting buffer (25 mM Tris, 200 mM glycine, pH 8.3, containing 20% methanol). The proteins were electrotransferred onto 0.45 um nitrocellulose filters (Schleicher and Schuell, Keene, NH) at 100 V for 1 to 1.5 h at 4°C in blotting buffer using the Transphor unit from Hoeffer (San Francisco, CA). The nitrocellulose filters were then incubated in milk solution (as described for RIA) for at least 1 h followed by an incubation for 2 h at room temperature with the appropriate antibodies diluted to 10 ug/ml in milk solution. When a bridging antibody such as rabbit anti-goat IgG (Sigma) was required, the filters were incubated with this antibody at a concentration of 20 ug/ml, for 1 h at room temperature. The final incubation was performed with either ^{125}I-goat anti-rabbit IgG or ^{125}I-rat anti-mouse IgG, at 2 x 10^6 cpm/ml milk solution for 1 h at room temperature. Multiple washes occured between incubations using several

changes of PBS/Tw20 and the final series was extensive. Bands were visualized by autoradiography on Kodak X-Omat RP film.

Results and Discussion

In order to determine whether any species cross-reactivity among the Hxs might be detected by these antibodies, the results obtained with three different techniques possessing a wide range of sensitivity were compared. Rocket immunoelectrophoresis, like double diffusion, is generally performed with micromolar antigen/antibody concentrations and measures only precipitable immune complexes usually precluding the use of McAbs. On the other hand, RIA and Western blotting detect non-precipitable immune complexes in the nanomolar range. RIA assays protein in its native conformation whereas Western blotting on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAG) analyses denatured protein and can verify immunospecificity. Therefore, the data obtained with these three techniques should be complementary.

The results are summarized in Table I and are expressed as relative percentages, assigning a value of 100% to the reaction of an antibody with the antigen to which it was specifically raised. Rocket immunoelectro-

TABLE I

Cross-reactivity of polyclonal and monoclonal antibodies against human, rat, rabbit, and chicken hemopexins

Antibody	Rocket Immunoelectrophoresis*				Solid-Phase Radioimmunoassay*				Western Blotting**			
	Human	Rat	Rabbit	Chicken	Human	Rat	Rabbit	Chicken	Human	Rat	Rabbit	Chicken
a) Polyclonal												
Rabbit α-human Hx	100	53	3	--	100	157	34	<1	++++	+++++	++++	+
Rabbit α-rat Hx	--	100	5	--	24	100	45	--	±	++++	±	±
Goat α-rabbit Hx	--	--	100	--	50	11	100	--	+	++	++++	+
Rabbit α-chicken Hx	--	--	--	100	13	11	6	100	--	±	--	++++
b) Monoclonal												
Mouse α-rat Hx (R4B3)		N/D			<1	100	10	--	--	++++	--	--
Mouse α-rabbit Hx (Rb3D11)		N/D			166	6	100	--	++++	--	++++	--
Mouse α-rabbit Hx (Rb3H9)		N/D			--	--	100	--	--	--	++++	--

* - Relative percentage: N/D, not determined
** - +++++ >100%; ++++ 100%; +++ ~50%; ++~25%; + 10%; ± <5%; -- 0

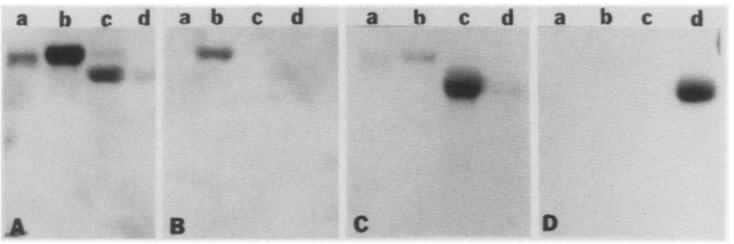

Figure 1. Human (lanes a), rat (lanes b), rabbit (lanes c) and chicken (lanes d) Hxs (5 ug each) were electrophoresed through 7.5-15% gradient $NaDodSO_4$-PAG and transferred onto nitrocellulose filter. Blots were incubated with the following PcAbs: A. rabbit anti-human Hx IgG, B. rabbit anti-rat Hx IgG, C. goat anti-rabbit Hx IgG, D. rabbit anti-chicken Hx IgG.

phoresis, employing anti-human PcAb, showed strong cross-reactivity with rat Hx and weak with rabbit Hx, and the anti-rat PcAb displayed minimal reactivity with rabbit Hx. These results are in agreement with those of Cox et al. (19), who used double diffusion in agar. As expected, RIA and Western blotting revealed a greater degree of cross-reactivity between the various antigens assessed with the PcAbs (Fig. 1). In general, the results obtained by these two techniques were comparable. There were two notable exceptions. The RIA conducted with PcAb anti-rabbit Hx showed a greater reaction with human than with rat antigen whereas this finding was reversed on Western blots. A similar seemingly conflicting result was seen in the degree of cross-reactivity with these two techniques between anti-rat Hx and rabbit Hx. These observations may reflect a substantially different epitope availability between native and denatured protein.

The extent of cross-reactivity was variable between all antibodies to mammalian Hxs and chicken Hx as well as between anti-chicken Hx and each of the 3 mammalian Hxs. Using immunoelectrophoresis, no cross-reactivity was observed. Cox et al. (19) also could not demonstrate immunoreactivity with an avian (duck) Hx using anti-human Hx PcAb. However, Western blotting with the PcAbs to the mammalian Hx clearly demonstrated the existence of some shared antigenic determinants with chicken Hx, although these are limited as judged by the relative intensity displayed in Fig. 1A-C and are not detected by RIA. This result may reflect the difference between chicken and mammalian

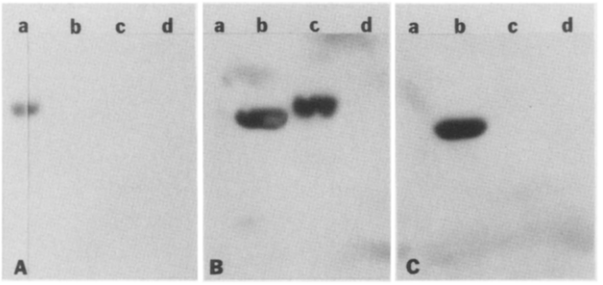

Figure 2. Western blot of rat, rabbit, chicken and human Hxs incubated with McAbs. Rat (lanes a), rabbit (lanes b), human (lanes c) and chicken (lanes d) Hxs (5 ug each) were electrophoresed through 7.5-15% gradient NaDodSO$_4$-PAG and transferred onto nitrocellulose filter. Blots were incubated with McAbs: A. R4B3, B. Rb3D11, C. Rb3H9.

Hxs (8) in amino acid composition, peptide chain length, the presumably extensive difference in sequence as well as the less complex carbohydrate content of the avian molecule.

The results obtained with the McAbs in RIA and Western blots were concordant. In Western blots R4B3 recognized only rat Hx, although rabbit and human Hxs were also minimally detected in the RIA (Table I and Fig. 2). With both techniques, Rb3D11 cross-reacted strongly with human Hx, whereas Rb3H9 only recognized the homologous protein (Table I and Fig. 2). These data indicate that these two McAbs are directed against different epitopes on rabbit Hx, one of which is common to human Hx.

When analysing data concerned with immunocross-reactivity the phylogenetic relationship between the species from which the antigens are derived as well as the species in which the antibodies are produced need to be taken into account. The similar temporal divergence of the mammalian species utilized in this study as source for antigens and antibodies would indicate that any sequence/structural diversity be the result of random mutation and probably would not occur in protein domains of selective importance (i.e. heme-binding, receptor interaction). The only conclusion that can be drawn from the present data is that enough structural resemblence exists among these species to consistently allow cross species immuno recognition. Whether the measurable degree of immunoreactivity between avian and mammalian

Hxs is due to retention of antigenic domains relatively resistent to random mutation is the subject of ongoing investigations.

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