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CHARACTERIZATION AND COMPARATIVE IMMUNOREACTIVITY OF ANTIBODY TO NEWT (*T. CRISTATUS*) GLOBINS

Louis A. Kowalski, Anne W. Walsh, Martha Merrow, Wayne Paulekus, William Mackin and Joseph A. Grasso

Department of Anatomy, University of Connecticut Health Center, Farmington, CT 06032, USA

Abstract—1. Rabbit antisera to newt (*T. cristatus*) globin were produced by repeated injections of globin and antiglobin antibodies purified by chromatography on globin–Sepharose 4B.

2. Ouchterlony and SDS PAGE analysis indicated that the material eluted from the affinity column was rabbit IgG.

3. The antiglobin antibodies tested by immunodiffusion and ELISA cross-reacted with native hemoglobin and globin from *T. cristatus* and to varying extents with globins of *N. viridescens*, *R. pipiens* and *X. laevis*, but not with human globin.

4. The degree of cross-reactivity appeared to parallel the evolutionary relatedness of these species, suggesting common antigenic determinants among globins of various vertebrate species.

INTRODUCTION

The cytological and molecular features of the erythropoietic process in the newt (*Triturus cristatus*) have been an object of study as a model for elucidation of regulatory mechanisms in RBC differentiation (Casale *et al.*, 1980). The cellular rate of hemoglobin synthesis, including production of globin chains and their assembly into tetrameric hemoglobin, declines progressively during the RBC developmental process (Casale *et al.*, 1980). Alterations in rates of protein synthesis may reflect the availability and integrity of *m*RNA as determined by transcriptional activity or may be imposed by primary changes at the translational level. The purpose of this study was to analyze the immunoreactivity of anti-newt globin antibody and to evaluate its immunospecificity as a prerequisite for its use in analysis of translation of globin peptides. Classical immunological methods have been employed to elicit and characterize polyclonal anti-newt (*T. cristatus*) globin antibodies. The high sensitivity of the enzyme linked immunosorbent assay (ELISA) has been used to determine the degree of antibody cross-reactivity among globins of closely related amphibia and man. This report describes the properties of antibody raised against newt globins, including its comparative reactivity to globin and hemoglobin from various amphibians.

MATERIALS AND METHODS

Preparation of antigens and antisera

Newt (*T. cristatus*) globins were isolated from washed erythrocytes by acid–acetone extraction (Rossi-Fanelli and Antonini, 1955). The purity of globin was confirmed by SDS gel electrophoresis, which revealed a single major band that co-migrated with pure globin. Antisera to newt globins were prepared by injecting 1 ml (1 mg/ml) of protein suspended in complete Freund's adjuvant (Calbiochem-Behring, La Jolla, CA) intradermally into New Zealand white rabbits. Intradermal boosts containing 1 mg antigen/ml in phos-

phate buffered saline pH 8.0 (PBS) and incomplete Freund's adjuvant were given biweekly for 1 month. Subsequent boosts were given in the flank intramuscularly every 14 days. Antisera were prepared from blood obtained 1 week after the booster injections and subsequently once a month.

Double immunodiffusion. Immunodiffusion experiments were done in agarose gel beds prepared on glass microscope slides previously coated with 0.1% Noble Agar (Difco Lab, Detroit, MI). The gel bed was formed by pouring melted 1.5% Noble Agar, in borate saline buffer (0.13 M NaCl, 0.16 M borate, pH 8.2) containing 2% polyethylene glycol (MW 6000, Sigma P2139), onto the slides (Eby *et al.*, 1973). The wells were filled with 10–25 μl of undiluted antiserum, or affinity purified IgG (2 mg/ml), and 10–20 μl of antigen solutions at various concentrations. After incubation at 100% humidity for 24 hr at ambient temperature, the slides were washed in several changes of 0.9% NaCl for 24 hr, covered with filter paper, and dried in an oven at 75 ℃ for 1–2 hr. The slides were stained for 15–45 min in 0.25% Coomassie Brillant Blue R-250 (Kodak 14013), dissolved in 50% methanol:10% acetic acid, and destained in acetic acid:methanol solution.

Immunoelectrophoretic techniques. Immunoelectrophoretic analysis was carried out using a modification of the method described by Teisberg (1970). The gel bed consisted of 1% agarose (Seakem Agarose, Marine Colloids) in 0.023 M barbital buffer (pH 8.65). Immune serum or column purified anti-newt globin IgG was applied to 1 mm antigen wells. After electrophoresis at a constant voltage of 150 mV for 2 hr, the gel was reacted with 100 μl of goat anti-rabbit whole serum (Sigma No. R4751) or goat anti-rabbit IgG (Sigma No. R3129). The gels were washed 10 times over a 48 hr period and stained as above.

Affinity chromatography. Cyanogen-bromide activated Sepharose 4B (Sigma) was washed and swollen on a sintered glass filter using 0.1 M HCl and gently mixed on a magnetic stirrer for 2 hr at room temperature with purified newt globin (5 mg/ml gel) dissolved in coupling buffer (0.1 M NaHCO₃, pH 8.3; 0.5 M NaCl). After pouring the column, removal of excess protein and blockage of remaining active groups on the gel bed was performed by washing with 0.1 M Tris buffer (pH 9.8). Alternate application of coupling buffer followed by 0.1 M acetate buffer (pH 4.0) was used to remove excess blocking reagent and adsorbed protein. Pooled immune serum was passed 4–10 times over a 7 cm

affinity column at a rate of 0.24 ml/min. The column was washed with BSB to remove non-specifically absorbed protein and the specific antiglobin IgG was eluted from the column with 0.2 M glycine–HCl. One ml fractions were collected and protein eluted from the column was detected by measuring absorbance at 280 nm. The IgG concentration was determined using an extinction coefficient $E^{1\%}_{1cm} = 15$ at 280 nm (Little and Donahue, 1968).

The antibody solution was adjusted to pH 8.0 and dialyzed for 48 hr against BSB at 4°C. The specificity and purity of the IgG were assayed on Ouchterlony plates and by immunoelectrophoresis and SDS gel electrophoresis.

ELISA

ELISA was performed by the method of Bullock and Walls (1977). Newt globin or hemoglobin dissolved in ELISA coating buffer (ECB), (0.05 M carbonate–bicarbonate buffer, pH 9.6) were added to each well of a LINBRO flat-bottomed microtiter plate (Flow Labs, McLean, VA, Cat. No. 76-381-04). For standardization of protein concentration of globin and hemoglobin, respectively, protein was measured by the Bradford method (Bradford, 1976), and diluted to contain 1 mg/ml. The plate was covered with parafilm, incubated at 37°C for 1 hr, and washed with PBS (pH 7.4) containing 0.5% v/v Tween 20 (PBS-T). Rabbit anti-T. cristatus globin sera, or the affinity column purified anti-T. cristatus globin IgG, were added to the appropriate wells. Control wells without antiserum contained PBS-T and T. cristatus globin or hemoglobin in ECB. The plates were incubated at 37°C for 60 min and washed as above. Peroxidase-conjugated goat anti-rabbit IgG (Per-Gar) (Cappel Labs, Cochranville, PA) was added to all wells (100 μl of 1:200 dilution) and the plates incubated for 60 min at 37°C. After washing with PBS-T peroxidase substrate (0.012, H_2O_2, 0.067% 2,2'-azino-di-3-ethyl-benzthiazoline sulfonate), 0.05 M citrate buffer was added to each well and the reaction allowed to proceed at ambient temperature. Absorbances were read at 414 nm on a Titertek Multiskan ELISA plate reader (Flow Labs Inc., McLean, VA).

Quantitation of relative cross-reactivity between anti-T. cristatus globin antibody and globins of other amphibian species

The ELISA curves derived from these experiments were plotted on graph paper and traced on layout vellum (Designer series No. 150 Beinfang Paper Co., Inc., Metuchen, NJ); the areas of the curves were carefully cut out and integrated by weighing on a Mettler Balance. The data, expressed as percent cross-reactivity, were normalized relative to the weight of the curves derived from the reaction of anti-T. cristatus globin IgG to purified T. cristatus globin as control.

Polyacrylamide gel electrophoresis

Affinity purified IgG and marker IgG (Sigma) were run on a 10% acrylamide-SDS slab gel (150 × 140 × 1.5 mm) with a 4% stacking gel (Cieplinski et al., 1981). Acid–acetone purified globins and purified IgG were analyzed on 5–22% gradient slab SDS gels (Gehrke et al., 1981). Samples were prepared for electrophoresis by adding 20–50 μl of protein (1–4 mg/ml) to 10–25 μl of 5× sample buffer (0.31 M Tris–HCl; 0.16 M H_3PO_4, 5% (w/v) SDS; 50 mM DTT; 2 mg bromophenol blue; pH 6.8) and heated at 100°C for 5 min. After heating, proteins were alkylated with iodoacetamide (Lane, 1978). The protein sample (20–50 μg) was loaded into the gel wells and electrophoresed at 50 V (constant voltage) until the ion front completely entered the stacking gel; the proteins were then electrophoresed at 40 mA (constant current) until the marker dye was 1 cm from the bottom of the gel (5–6 hr).

RESULTS

Antisera to newt (T. cristatus) globin at titers detectable by double immunodiffusion techniques were obtained approx 5 weeks after the initial immunization. Two to three intramuscular boosts were required before precipitin patterns could be visualized. The titer of anti-newt globin antibody in immune serum was monitored over an 8-month period using the ELISA assay. A relatively high titer (1:16) of antibody was present after three intramuscular boosts and was maintained in the serum for approx 3 months (Fig. 1). In the fourth month, the titer began to drop so that additional intramuscular boosts of globin in incomplete Freunds (1:1) were given. After these boosts, the level of anti-newt globin antibody rose by 25%, this titer being maintained over the next year by monthly boosts of antigen.

Antisera tested on an immunodiffusion plate against purified newt globin yielded a definite but diffuse precipitin line, suggesting the presence of heterogeneous antibodies interacting with the purified globin (Fig. 2). To increase antibody purity, affinity chromatography of the immune antiserum was carried out on a globin–Sepharose 4B column. One major peak of protein was eluted from the column (Fig. 3), which, when tested on an immunodiffusion plate against purified newt globin, yielded a crisp, sharp precipitin reaction (Fig. 4).
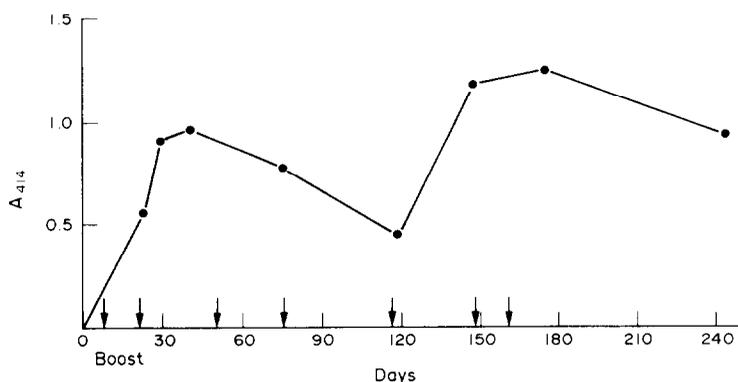

Fig. 1. Graph depicting the titer of anti-newt globin antibody in immune serum determined over an 8-month period by the ELISA assay. The inverted arrowheads on abscissa indicate times of boosts.
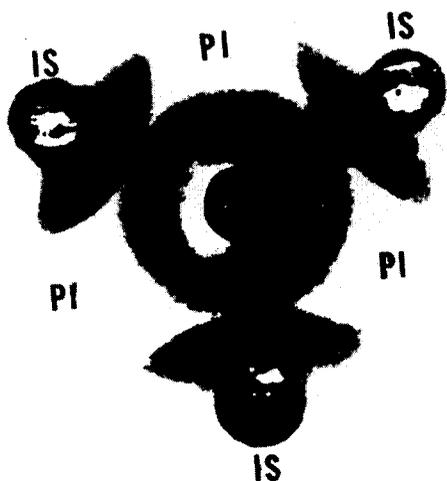
Fig. 2. Immunodiffusion plate of unfractionated rabbit anti-*T. cristatus* globin serum against purified *T. cristatus* globin (center well). IS, immune serum; PI, pre-immune serum. Plate stained with Coomassie Blue.

indicating a highly purified preparation of anti-newt globin antibody.

The identity of the affinity purified fraction as IgG was confirmed by immunoelectrophoresis which revealed multiple precipitin lines upon reaction against goat anti-rabbit serum antisera and a single band when reacted against goat anti-rabbit IgG (Fig. 5). With SDS–acrylamide gel electrophoresis, the affinity purified IgG co-electrophoresed with marker IgG under non-reducing conditions, appearing as a sharp distinct band after staining with Coomassie Blue (Fig. 6). Following reduction and alkylation, and electrophoresis on SDS–polyacrylamide gradient gels, the affinity purified fraction yielded a major band in the region corresponding to $M_R = 50,000$
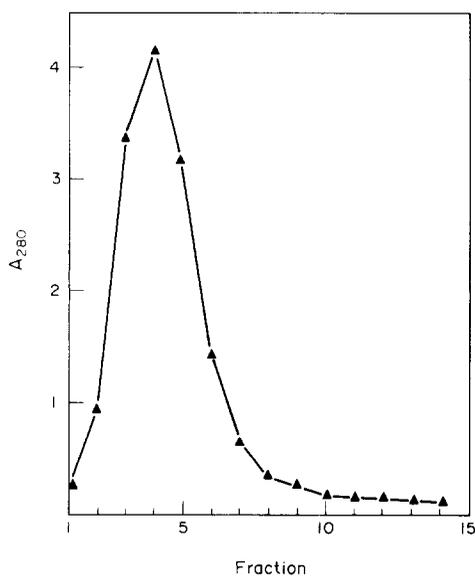
daltons (heavy chains) and a pair of less intense bands (light chains) in the region corresponding to $M_R = 25,000$ daltons. These values agree with previous estimates of the molecular weights of heavy and light chains of rabbit IgG (Ahmad-Zadeh *et al.*, 1971) and support the identity of the affinity purified fraction as IgG.

No reactivity of antibody was detected against other endogenous proteins of newt erythroid cells; the antibody failed to react with ferritin or monoribosomes. However, antisera effectively precipitated polyribosomes isolated from newt erythroid cells, most likely due to the presence of nascent globin peptides associated with the polysomes.

Since both hemoglobin and free globin chains are present in newt erythroid cell lysates (Casale *et al.*, 1980), the extent of reactivity of anti-*T. cristatus* globin IgG to tetrameric hemoglobin was determined. For this purpose, a novel, rapid enzyme-linked immunosorbant assay (ELISA) was devised. When equal concentrations of *T. cristatus* globin and native hemoglobin were reacted with increasing concentrations of affinity purified IgG ranging between 10 and 10,000 ng/ml, two non-overlapping curves were generated (Fig. 7). The anti-newt globin IgG reacted with hemoglobin at a concentration 10-fold lower than its reaction to globin. The curves for hemoglobin and globin reached a plateau at an IgG concentration of 10^5 ng/ml.

The degree of cross-reactivity between anti-*T. cristatus* globin IgG and globins from related species and man was also examined. Globin $(10 \,\mu g)$ was passively absorbed to microtiter plates and reacted with column purified anti-*T. cristatus* globin IgG at concentrations ranging from 0.8 up to $100 \,\mu g/ml$. The reaction of anti-*T. cristatus* globin IgG to *T. cristatus* globin repeatedly reached a plateau at $25 \,\mu g/ml$ IgG in three separate trials (Fig. 8). Integration of the area beneath each ELISA curve was performed and relative cross-reactivity determined. Using this method, the relative proportion of cross-

Fraction

Fig. 3. Elution pattern of anti-globin IgG purified by affinity chromatography on globin–Sepharose 4B.
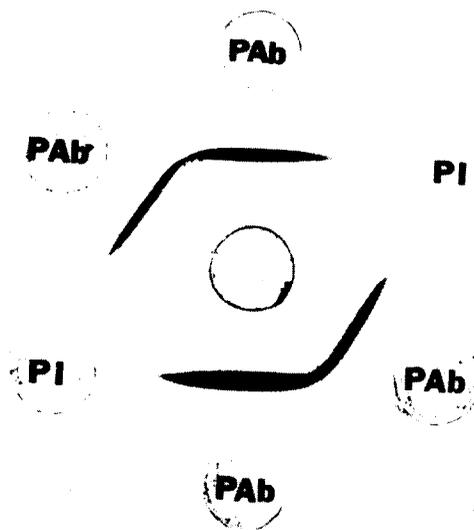

Fig. 4. Immunodiffusion plate showing reaction of purified anti-*T. cristatus* globin IgG (PAb) against *T. cristatus* globin. Control wells containing pre-immune serum (PI) show no reaction to antigen.
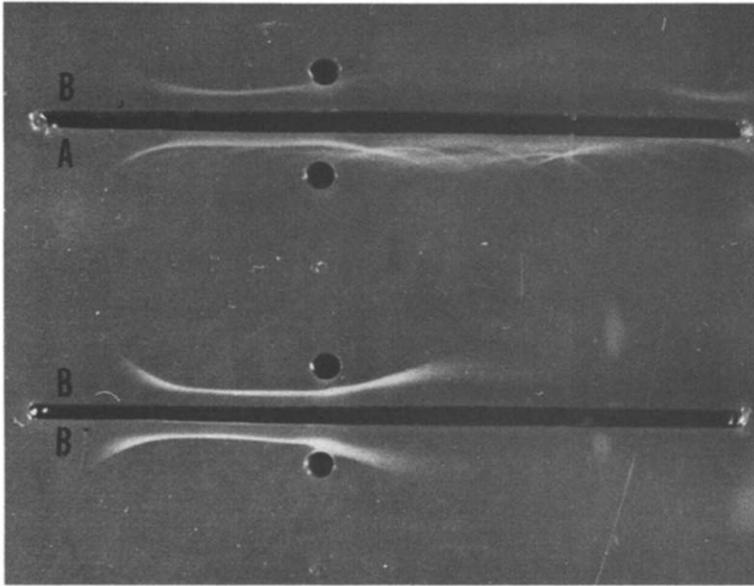
Fig. 5. Immunoelectrophoresis of whole immune serum (A) and affinity column purified rabbit anti-newt globin IgG (B) reacted with goat anti-whole rabbit serum (upper trough) or goat anti-rabbit IgG (lower trough).
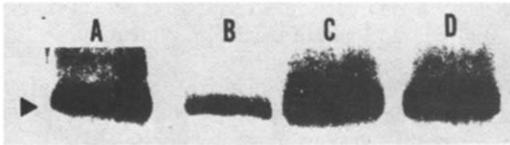

Fig. 6. SDS-10% acrylamide gel electrophoresis of marker rabbit IgG (lane A, 15 μg, lane B, 5 μg) and column purified anti-*T. cristatus* globin IgG (lanes C and D).

reactivity between anti-*T. cristatus* IgG and non-homologous globins of various species was *N. viridescens*, 73%; *R. pipiens*, 33%, and *Xenopus laevis*,

25% (Table 1). No cross-reactivity was demonstrated between anti-*T. cristatus* globin IgG and isolated human globin chains (Table 1).

DISCUSSION

This study presents data describing the successful production and characterization of antibody to globin and hemoglobin of the newt, *T. cristatus*. Although previous studies have described the synthesis of antibody to hemoglobin and globin (Maniatis and Ingram, 1971; Paluska et al., 1976; Chapman and Tobin, 1979; Just et al., 1980; Flavin
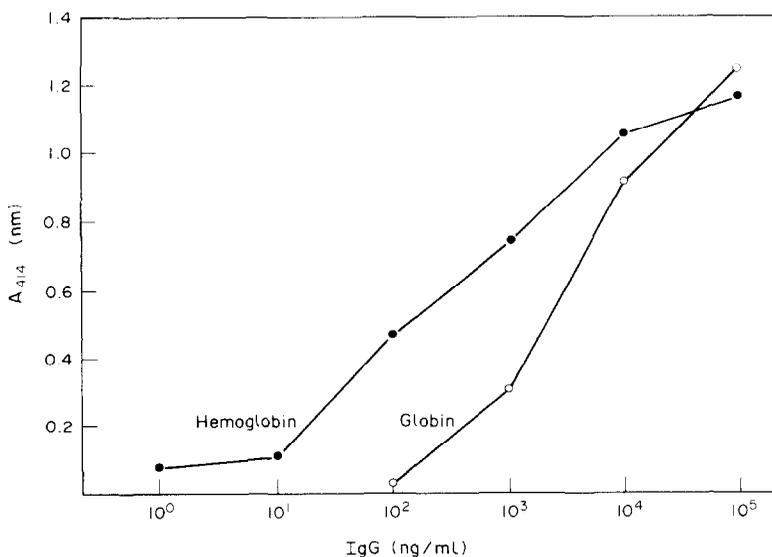

Fig. 7. Relative reactivity of globin (O) and native hemoglobin (●) with affinity purified rabbit anti-*T. cristatus* globin IgG determined by ELISA. Equal amounts of globin and hemoglobin were reacted with increasing concentrations of anti-globin IgG.
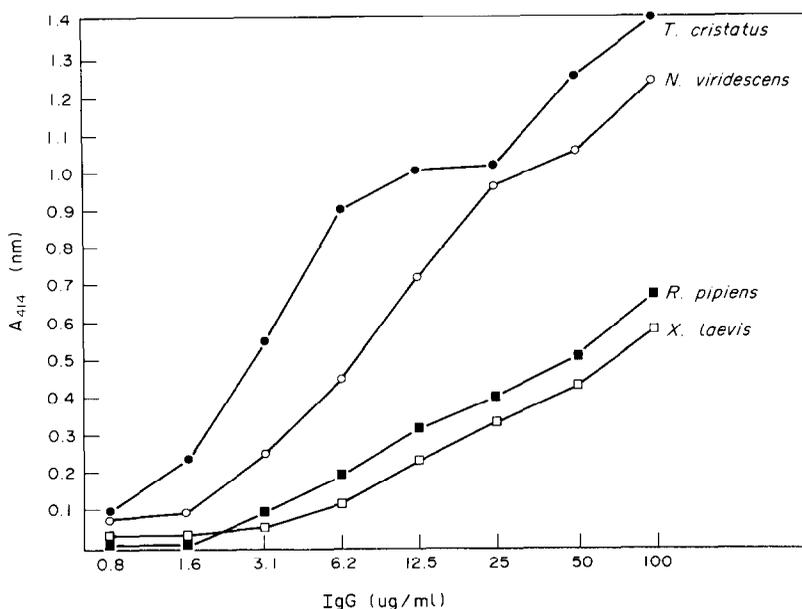
Fig. 8. ELISA analysis showing kinetics of binding of purfied rabbit anti-*T. cristatus* globin IgG to globins isolated from various species.

et al., 1982), none has monitored the immune response from its inception through achievement of a maximal titer. Our studies have shown that it is difficult to obtain a high titer antibody to globin, even after multiple intramuscular boosts. This may possibly be explained by the fact that hemoglobin is a highly conserved molecule throughout the vertebrate phylum (Bunn, 1981) so that the immune system of the rabbit resists production of antibody to various antigenic determinants in newt globin which may be homologous to determinants in rabbit globin. Other studies on the antigenic nature of human globins have suggested this possible explanation for the relatively weak antigenicity of globin (Reichlin, 1972).

In monitoring the appearance of anti-newt IgG during immunization of the rabbit, two plateaus of IgG are detected by ELISA over the 240-day immunization period (Fig. 1). A similar biphasic plateau pattern is obtained in the binding of purified anti-*T. cristatus* globin IgG to pure globin (Fig. 7). It is well recognized that, during immunization, increasing the number of boosts of antigen elicits increased recognition of antigenic determinants. In view of this

Table 1. Relative cross-reactivity between anti-*T. Cristatus* globin antibody and other amphibian species

Species	Relative % cross-reactivity
T. cristatus	
N. viridescens	73.2
R. pipiens	32.6
X. laevis	25.2
Man	0

Data from ELISA assay procedure was plotted on graph paper; curves depicting reactivity of antibody with globins of designated species were traced, cut out and weighed on an analytical balance to integrate the area under the curves.

relationship between antigenic dosage and recognition, our results suggest that newt globins possess a major and a minor determinant which are sequentially recognized during the immune reaction in the rabbit.

Makler and Pesce (1980) have shown that the form of an antigen influences the amount of antibody reacting with it. Their studies have shown that curves obtained for reaction of ant-HbA with human HbA and β-globins, respectively, were superimposable while two non-superimposable curves were obtained for HbF and the reactions between anti-λ globin with pure λ globin. Our results on the binding of anti-*T. cristatus* globin antibody to globin and hemoglobin yield two non-superimposable curves in which the binding of antibody to hemoglobin occurs at a concentration 10-fold less than that required for binding to globin (Fig. 7). This difference in binding may be explained by assuming that globin and hemoglobin may bind unequally to the microtiter plate with more hemoglobin than globin retained after application. However, the same result was obtained several times; moreover, other laboratories have reported equal binding of human globin and hemoglobins under conditions identical to those used in these studies (Makler and Pesce, 1980). A more likely explanation for the difference in binding is that native tetrameric hemoglobin molecules may contain more antigenic determinants which are accessible to the antibody than are represented in monomeric globin.

These results showing dissimilar reactivity between antibody and native hemoglobin and globin, respectively, do not support the hypothesis described by Sela *et al.* (1967). These authors reported that antigenic determinants in protein molecules are of two types: sequential determinants consisting of primary structure and conformational determinants im-

posed by secondary and higher levels of protein structure. According to this hypothesis, antibodies prepared against native proteins will react weakly against denatured protein while antibodies raised against denatured protein will bind weakly against native antigen. Our results on the binding of anti-*T. cristatus* globin antibody to globin and hemoglobin, respectively, do not correlate with the results expected from the hypothesis; instead, antibody prepared with denatured globin reacted to native hemoglobin at an antibody concentration 10 × lower than that required for reaction with denatured globin. This result suggests that the antibody recognizes conformational determinants in native hemoglobin preferentially over the sequential determinants in denatured globin or that the increased presence of antigenic determinants in tetrameric hemoglobin may cancel out the expected effect. However, even assuming that the native molecule can contain four times the number of antigenic determinants present in monomeric globin, the reactivity of antibody against native hemoglobin is still higher than that obtained against denatured globin.

Although the protein sequence and structure of vertebrate globins have been compiled and contrasted for a large number of vertebrate and invertebrate globins (Dayhoff *et al.*, 1972), few studies have examined the inter-relationship of closely related amphibian globins immunologically. The structural differences among homologous proteins of closely related species were first revealed by antibody antigen reactions (Landsteiner, 1962). Our results, using anti-*T. cristatus* globin antibody to examine cross-reactivity to globin from closely related amphibian and man, mirror the phylogenetic relationship of the species examined. When testing for cross-reactivity using double immunodiffusion, spur formation was seen only in the reaction of anti-*T. cristatus* globin IgG to *N. viridescens* globin (unpublished data). No cross-reactivity could be demonstrated by this technique to either of the two anuran globins or human globins. In other studies employing double immunodiffusion (Paluska *et al.*, 1976), antisera prepared against human and canine globins cross-reacted with heterologous globin on immunodiffusion plates but the antisera failed to cross-react with rabbit, bovine or horse globins.

Re-analysis of relative cross-reactivity using a more sensitive, quantitative technique (ELISA) demonstrated cross-reaction of antibody to globins of four amphibian species at proportions which generally correlate with the degree of phylogenetic relationship. Thus, greater cross-reactivity of anti-*T. cristatus* IgG was seen against globins of a closely related species, the North American newt (*N. viridescens*) while considerably less reactivity was observed with anuran globins. No cross-reactivity was seen with human globin.

These results indicate a degree of immunological relatedness among different amphibian globins which is obscure when analyzed by classical immunological assay methods but readily resolved by the more sensitive ELISA assay. The extent to which such ELISA reactivity indicates the existence of shared antigenic sequences among the various globins has not been determined. Nevertheless, these results and

the availability of the highly sensitive ELISA assay raise the need for caution in immunochemical analyses of globins where failure to exclude cross-reactivity even among globins of the same species could lead to misinterpretation of experimental data. The application of this specific, well-characterized antibody in analysis of molecular events regulating hemoglobin synthesis during newt RBC production will be reported elsewhere (Kowalski *et al.*, unpublished data).

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