The Presence of Two Antigenically Distinct Light Chains (κ and λ?) in Alligator Immunoglobulins[1] (34475)

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Current data regarding the phylogenetic development of immunoglobulin structure and function indicates that elasmobranchs have 19S and 7S immunoglobulins, each of which can be considered analogous to the IgM class (1–5). However, the 19S and 7S immunoglobulins of amphibians resemble IgM and IgG respectively (6). In order to establish the phylogenetic level at which additional classes of immunoglobulins appeared, the Florida alligator, (*Alligator mississippiensis*), a representitive of the class reptilia, was chosen. Initial studies with "early" immune sera to *Salmonella typhimurium* resulted in the purification of a 19S mcacroglobulin which contained agglutinating antibodies. This protein will subsequently be referred to as 19S Ig.[3] A 7S fraction containing two proteins of identical "IgG-like" electrophoretic mobilities was also isolated.

The purpose of this paper is to present evidence (a) that the proteins in this 7S fraction belong to one immunoglobulin class with two antigenically distinct L-chain types, and (b) that these two L-chains are probably under the control of different genetic loci.

Materials and Methods. Animals. Young alligators were used (41-56 in. in length), and sera were obtained from the caudal vessels.

Fractionation procedures. Alligator sera to be chromatographed on diethylaminoethyl–cellulose (DEAE–cellulose) were dialyzed against 0.015 *M* Tris (hydroxymethyl)-aminomethane (Tris) buffer, pH 8.0, overnight. DEAE-cellulose chromatography (Whatman, DE32) was performed at room temperature with an increasing NaCl gradient to elute absorbed protein (7). Gel filtration (8) was achieved with Sephadex G200 in buffer consisting of 0.14 *M* NaCl, 0.01 *M* Tris-HCl, and 0.001 *M* ethylene-diaminetetraacetic acid (EDTA) at pH 7.4 (Sephadex buffer).

Partially reduced heavy and light polypeptide chains (H- and L-chains) were separated on Sephadex G200 columns equilibrated with 5 *M* guanidine-HCl, as described by Lamm *et al.* (9). Briefly, this consisted of partial reduction for 1 hr at room temperature in 0.1 *M* dithioerythritol buffered at pH 8.2 with 0.46 *M* Tris-HCl followed by alkylation at 0°C with 0.22 *M* iodacetamide. The partially reduced and alkylated protein was then dialyzed twice against 100 vol of Sephadex buffer for 12 hr at 4° prior to being adjusted to 5.3 *M* guanidine–HCl and applied to an upward flow Sephadex G200 column equilibrated with 5 *M* guanidine–HCl.

A Beckman DB spectrophotometer was employed to determine the presence of protein by optical density at 280 mμ (assumed $\epsilon^{1\%}_{280,\,1\,cm} = 14.0$). Appropriate fractions were concentrated by positive-pressure dialysis.

Sedimentation coefficients were obtained in a Beckman model E analytical ultracentrifuge as described by Schachman (10).

Preparation of antisera. Rabbit antisera to alligator serum and purified polypeptide chains were as follows. The respective anti-

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[3] Unpublished results of J. Krauss, L. W. Clem, and P. A. Small from this laboratory. The 19S immunoglobulin resembled IgM based on sedimentation coefficients and molecular weights and antigenic distinctiveness of H-chains relative to other serum proteins.

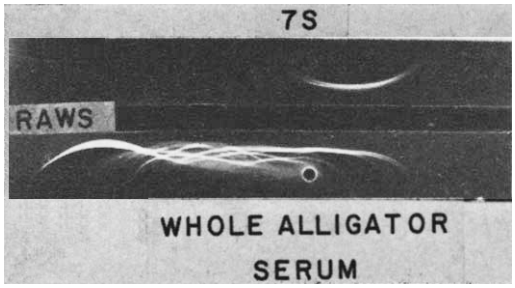

FIG. 1. Immunoelectrophoresis showing "IgG-like" mobilities and double precipitin arc associated with the isolated 7S fraction and whole alligator serum. The concentration of the 7S fraction was 4.75 mg/ml. The anode is to the left.

gens (undiluted serum or 0.25 mg/ml for the chains) were emulsified 1:1 with Freund's complete adjuvant (Difco) and 0.1 ml of emulsion was injected into each hind foot pad and two 0.4-ml aliquots were injected subcutaneously on the animals' back. Subcutaneous 0.1-ml injections on the back were repeated at 2 and 4 weeks. The strength and purity of the antisera were defined qualitatively by Ouchterlony analysis and immunoelectrophoresis performed as previously described (3).

Results. DEAE-cellulose ion-exchange chromatography of pooled alligator serum yielded an initial fraction eluting with the equilibrating buffer. This material was monodisperse in the ultracentrifuge with sedimentation coefficients of 6.8S and 7.1S at concentrations of 3.0 and 1.5 mg/ml. This material will hereafter be referred to as 7S. In some experiments this fraction contained small amounts of macroglobulin which was removed by Sephadex G200 gel filtration. Most of the alligator 19S immunoglobulin was eluted from DEAE-cellulose columns soon after the gradient was started; this immunoglobulin was further purified by Sephadex G200 gel filtration.

Immunoelectrophoresis of the 7S fraction with the 74th day bleeding from rabbit No. 126 previously immunized with whole alligator sera (hereafter referred to as RAWS) revealed the presence of two precipitin arcs of identical "IgG-like" mobilities (Fig. 1). Also shown in this figure is whole alligator serum wherein these two arcs are visible (most cathodic mobilities). Sera from earlier bleedings from this rabbit and from two other rabbits detected only one precipitin arc against the 7S fraction by immunoelectrophoresis.

This 7S fraction was subjected to partial reduction and alkylation followed by gel filtration on a Sephadex G200 column equilibrated with 5 *M* guanidine–HCl. The elution profile and elution volumes were similar to those observed in other experiments using IgG from other species; *i.e.*, about 75% heavy and 25% light material. For identification purposes these will be referred to as 7S H- and L-chains. Rabbit antisera to each of the 7S polypeptide chains were prepared and Fig. 2 shows the results of an immunodiffusion pattern obtained by using antisera against 7S H- and L-chains and the respective isolated chains. Several points are evident: (a) the anti-7S L-chain serum shows two precipitin bands with 7S L-chains but does not react with 7S H-chains, and (b) only one band is evident between the 7S H-chain and its specific antiserum. Additional Ouchterlony analysis (not shown) using RAWS showed one precipitin band with the 7S H-chain preparation and two bands with the 7S L-chain preparation comparable to Fig. 2; the H-chains were antigenically distinct from the L-chains. These results suggested that two antigenically distinct L-chains may be responsible for the initial observation of a double precipitin arc observed in the 7S fraction against RAWS via immunoelectrophoresis (Fig. 1). This suggestion was further substantiated by the following experiment. Two

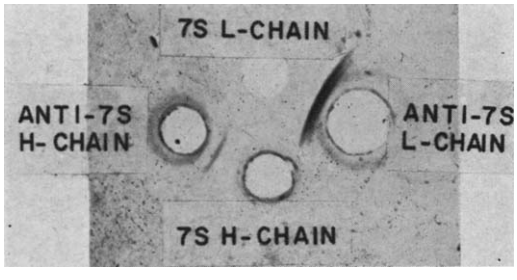

FIG. 2. Immunodiffusion pattern of isolated 7S polypeptide chains and their respective specific antisera. The concentration of the 7S H-chains was about 1.4 mg/ml and of the 7S L-chains about 0.8 mg/ml.

samples of RAWS were absorbed with isolated polypeptide chains from the 7S protein according to the following procedure. One milliliter of RAWS was mixed with 0.5 ml of the appropriate isolated polypeptide chain (either H- or L-chains at $OD_{280,\ m\mu} = 0.63$) and placed in 37° water bath for 1 hr with frequent stirring. The mixture was then placed in the cold for 24 hr. The resulting precipitate was pelleted by centrifugation and the supernatant fraction checked via immunoelectrophoresis and Ouchterlony analysis for effectiveness of absorption. It was found that RAWS absorbed with 7S H-chains and RAWS absorbed with 7S L-chains were unreactive against 7S H-chains and 7S L-chains respectively. The former absorbed serum will hereafter be referred to as RAWS(H) and the latter as RAWS(L). Immunoelectrophoresis of the intact 7S molecule against the absorbed antisera, RAWS(H) or RAWS(L) was performed analogous to Fig. 1. RAWS(H) did not react with 7S H-chains (showing that absorption removed the anti-7S H-chain activity) but showed two antigenically distinct precipitin arcs against the intact 7S molecule; whereas RAWS(L) yielded only a single precipitin arc against the intact 7S molecule while remaining unreactive with 7S L-chains. This single arc presumably resulted from the anti-7S H-chain activity of RAWS(L) since the latter gave no reaction against the 7S L-chains. In order to insure that these results were not artifacts resulting from the dilution of RAWS by the absorption procedure, a control was utilized in which RAWS was appropriately diluted with buffer alone. By immunoelectrophoresis, RAWS (diluted) was shown to be unaffected since the double precipitin arcs were indistinguishable from that observed with RAWS (undiluted) and both yielded the expected precipitin arcs against isolated 7S polypeptide chains.

The establishment of the 7S protein fraction as an immunoglobulin was performed as follows. Rabbit antiserum made against 19S L-chains (the original 19S fraction contained antibody activity) reacted with 7S L-chains yielding two precipitin bands as did the anti-7S L-chain antisera depicted in Fig. 2. Antisera to 7S H-chains did not react with 19S H-chains and vice versa.

Since the alligator sera used in the above studies were pooled, it was impossible to know whether the two L-chain variants were allotypes or whether they were different L-chain types and therefore controlled by nonallelic genes. If there existed allotypic differences in the L-chains, one would expect to see some alligators with both variants of L-chains and some with only one. If there were truly two types of L-chains, one would expect each individual to have both. The 7S fraction was isolated from the sera of six individuals captured at different times and at different places in North Central Florida and tested for the presence of the double precipitin arc by immunoelectrophoresis as in Fig. 1. Five of the alligators observed clearly exhibited this phenomenon. Interpretation of the Ouchterlony pattern of the sixth alligator was difficult due to a diffuse, broad band which made it impractical to assign to either a single or double pattern. Inadequate amounts of material prohibited further analysis; however, two arcs (of slowest mobilities) were clearly visible by immunoelectrophoresis using the original serum from this alligator comparable to Fig. 1. Nevertheless, only five alligators will be considered for the following analysis. The probability of observing five of five alligators with the two precipitin arcs assuming the hypothesis that the L-chains were allotypes is $(1/2)^5$, or 0.031. (see Discussion). Therefore, it seemed improbable that the banding was due to allotypic differences; rather, the L-chains were of two distinct types, presumably controlled by nonallelic genes.

Discussion. The data presented indicated that the isolated 7S fraction from alligator sera contained one immunoglobulin class with two antigenically distinct L-chain types probably controlled by separate genetic loci. This was first detected with RAWS (Fig. 1). It was fortuitous that rabbit No. 126 was able to pick up the L-chain determinants in alligator whole sera. None of the other rabbits immunized with whole serum were able to do so.

This 7S immunoglobulin belonged to a dif-

ferent immunoglobulin class than 19S Ig based on antigenic distinctiveness of the respective H-chains. Based upon its immunoelectrophoretic mobility and its sedimentation coefficient, it is tempting to refer to this 7S molecule as IgG. However, it is felt that additional criteria, such as molecular weights of polypeptide chains and carbohydrate content, must be determined before this designation can be correctly made. For a more thorough discussion of the classification of lower vertebrate immunoglobulins, the reader is referred to a recent review (11). 19S Ig also contained two L-chain types as shown by two precipitin bands between anti-19S L-chains and 7S L-chains. A point that should be mentioned is that the intact 19S Ig molecule showed only one precipitin arc with antiserum specific for L-chains as well as one precipitin arc with the same antiserum against isolated 19S L-chains. This may be due to a superimposition of the two precipitin lines.

Based on observing five of five alligators with both L-chain types, it seemed reasonable that these represented L-chains controlled by independently segregating loci. There are two main hypotheses to explain these observations. One is that the two L-chain bands represent proteins controlled by two separate loci comparable to κ and λ L-chains in man and therefore would be found in all normal individuals. The other hypothesis is that they are the product of two allelic genes at one locus and hence both would be present only in the heterozygote. Considering the latter hypothesis and assuming equal gene frequencies one would expect one half of the animals to show both bands while one quarter would how one of the bands and one quarter the other. This is the least favorable assumption, since unequal gene frequencies would result in less than half the animals being heterozygotes and, therefore, less than half of the animals would exhibit both bands (12). Then, if the assumption of equal gene frequencies is sufficient to reject the hypothesis that the L-chains are products of allelic genes, certainly the same is true of unequal gene frequencies. If we focus on the double bands only (the hypothesized heterozygote), the probability of observing, by chance, five such animals sequentially is $(1/2)^5$ or 0.031. Since this probability is relatively low, it is felt that the two L-chain types probably represent products of separate loci.

Although no direct evidence is presented here that the two L-chains were analogous to the kappa and lambda types of higher animals (13), several observations support such an interpretation. The L-chains were found to be antigenically distinct with identical electrophoretic mobilities. They were also shown to be controlled by genes which are probably not allelic. Each class of immunoglobulin, 19S and 7S, possessed both types of L-chains. Inasmuch as mammals and birds, the two classes of higher vertebrates which evolved separately from reptiles, each have kappa and lambda types of L-chains, it was reasonable to suspect that reptiles might also possess two types, as suggested by Hood *et al.* (14). Thus, our findings suggest that kappa and lambda L-chains found in higher vertebrates may have evolved at or prior to the phylogenetic level of the reptiles.

Summary. Alligators have been shown to possess two antigenically distinct L-chain types which are probably under the control of independently segregating genes perhaps comparable to κ and λ in man. The relevance of these findings with respect to the evolution of L-chain types was briefly discussed.

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