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Immune response in the garter snake (*Thamnophis ordinoides*)

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Summary. Garter snakes (*Thamnophis ordinoides*) were immunized with hen egg albumin, human gamma-globulin and Keyhole limpet haemocyanin in Freund's adjuvant. Antibody was consistently detected by radioimmunoelectrophoresis and in three different γ- and β-globulin precipitin lines called Ig-M ($\simeq$20S), Ig-1 ($\simeq$9S) and Ig-2 ($\simeq$8·5S). Early antibody (day 31 after immunization) was frequently Ig-M whereas Ig-2 and especially Ig-1 were detectable for the longest duration (992 days). After immunization with antigen in Freund's adjuvant, Ig-1 serum concentration showed the greatest increase, from almost undetectable levels to the most prominent immunoglobulin in immune serum.

INTRODUCTION

The immune response in lower vertebrates is of interest not only because of its evolutionary implications to mammalian immunology, but also because of the potential for a simplified immune system with unique advantages as an experimental model. In regard to the latter possibility, the immunoglobulin (Ig) heterogeneity in lower vertebrates has been found to be remarkably complex with multiple Ig classes evident at the level of amphibians (Marchalonis & Edelman, 1966; Lykakis & Cox, 1968; Coe & Peel, 1970; Acton, Evans, Weinheimer, Niedermeier & Bennett, 1972); that is, antigenically distinct low molecular weight (LMW) Ig of 7–8S are found in addition to the high molecular weight (HMW) Ig which is detectable at the level of primitive cyclostomes (Marchalonis & Edelman, 1968; Thoenes & Hildemann, 1970). Whereas only one LMW Ig class is present in amphibians, more than one antigenically or molecular-size distinct LMW Ig have been detected in reptiles such as turtles (Ambrosius, Hemmerling, Richter & Schimke, 1971; Coe, 1972; Leslie & Clem, 1972).

The present report concerns the Ig in snakes, a reptile whose immunological capacity is virtually unknown. Using the garter snake as a model, one HMW Ig-M and two LMW Ig were identified. One of the LMW Ig was almost undetectable in normal serum, but contained the most antibody for the longest interval after immunization with antigen in Freund's adjuvant.

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MATERIALS AND METHODS

Animals

New Zealand white rabbits were obtained locally.

Garter snakes (*Thamnophis ordinoides*) were obtained locally and from Bear Wildlife Refuge, Brigham City, Utah. Garter snakes (GS) were numbered with a metal band in the lower jaw and caged in a room maintained at 80°F. The snakes were fed 1-day-old mice weekly and water was available *ad libitum*. Plasma samples were obtained from an incision in the tail.

Antigen and antibody determination

Hen egg albumin (HEA) from K & K Laboratories Incorporated, Jamaica, New York was labelled with ^{131}I from ICN as previously described (Coe, 1968). Rabbit gamma-globulin (RGG) and human-gamma glubulin (HGG) from Pentex Incorporated, Kankakee, Illinois and dissociated (Weigle, 1964) Keyhole limpet haemocyanin (KLH) from Pacific BioMarine Supply Company, Venice, California were labelled with ^{131}I or ^{125}I using chloramine-T (McConahey & Dixon, 1966). Antibody activity was detected by radioimmunoelectrophoresis (RIEP) as previously described (Coe, 1968) using rabbit anti-whole snake antiserum to develop the immuno-electrophoretic (IEP) patterns.

Inoculation

Snakes were inoculated intraperitoneally, initially with 1 ml of Freund's complete adjuvant (FCA) (Difco) emulsion containing 0·5 mg antigen and then, at various intervals (31, 56 and 122 days later) with 0·5 mg antigen in 1 ml Freund's incomplete adjuvant (FIA) emulsion.

Rabbits were inoculated in footpads with antigen emulsified in FCA and commencing 3 weeks later then received three weekly injections of antigen in FIA.

Other studies

Normal or immune snake serum was filtered through Sephadex G-200 (Pharmacia, Uppsala, Sweden) and electrophoresed on a Pevikon block as previously described (Coe, 1968). Linear 10–44 per cent sucrose density gradients were prepared (Coe, 1968) using ^{125}I-labelled RGG and ^{131}I-labelled HTG (hog thyroglobulin) (Pentex Incorporated, Kankakee, Illinois) for 7S and 19S markers respectively. Fractions were removed from the top of the gradient with an Isco fractometer (Lincoln, Nebraska). Quantification of Ig in individual fractions was done according to Laurell (1966), using rabbit antisera specific for GS Ig-1 and Ig-M. The results are presented in arbitrary concentration units relative to a standard normal serum.

Purified garter snake antibody was obtained by absorbing serum from HEA-immunized snakes onto columns containing Sepharose 2B (Pharmacia, Uppsala, Sweden) conjugated by cyanogen bromide (Cuatrecasas, 1970) to HEA. After washing, antibody was eluted with 0·5 M acetic acid, neutralized with Tris base, dialysed against phosphate-buffered saline, and concentrated by negative pressure dialysis. This garter snake anti-HEA was injected into rabbits to produce rabbit anti-garter snake Ig (anti-GS Ig).

Quantitative estimates of Ig in serum of normal and immune snakes were done by testing two-fold

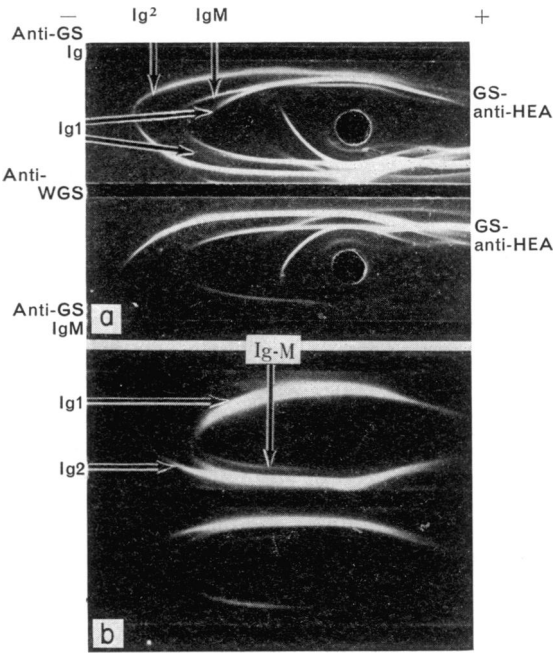

Figure 1. Immunoelectrophoresis slide (a) and respective ^{131}I-labelled HEA autoradiograph (b) of a garter snake serum obtained 88 days after primary immunization. Rabbit anti-garter snake Ig (top trough, anti-GS Ig) precipitates three Ig lines called (from cathode to anode) Ig-2, Ig-M and Ig-1. The Ig-2 and Ig-M lines pecipitated by anti-whole garter snake serum (middle trough, anti-WGS) are seen to fuse with the respective precipitins produced by anti-GS Ig. However, the Ig-1 precipitin of anti-WGS is a faint spur between the Ig-2 and Ig-M lines. The relationship between the Ig-1 precipitin lines of anti-GS Ig and anti-WGS can be appreciated only by the amplification on the autoradiograph below (see also Fig. 6). The bottom trough of IEP slide contains rabbit anti-GS Ig-M which was produced by immunizing rabbits with whole GS bile; the antiserum shown was unabsorbed, yet did not cross-react with Ig-1 and Ig-2.

dilutions of serum for endpoint precipitins in agarose diffusion with specific antisera.

Analytical ultracentrifugation of whole snake serum diluted with PBS to a protein concentration of 5 mg protein/ml was performed in a Spinco Model E ultracentrifuge at 20° and 50,740 r.p.m.

RESULTS

Radioimmunoelectrophoresis of serum obtained from immunized snakes revealed specific binding of ^{131}I-labelled antigen in three distinct precipitin lines. Fig. 1 shows an IEP of HEA-immune snake sera (two top wells) and the respective ^{131}I-labelled HEA autoradiograph below. Rabbit anti-GS Ig (Fig. 1, top trough) precipitated the following Ig precipitin lines: (1) the most cathodal outer line was called Ig-2 and fused with the outer line precipitated by anti-whole garter snake serum (anti-WGS, middle IEP trough); (2) the next most cathodal precipitin line corresponded to HMW-Ig-M and fused with the inner precipitin line of anti-WGS; (3) the third precipitin line (called Ig-1) corresponded to a faint spur produced by anti-WGS between the Ig-2 and Ig-M precipitin lines, a relationship difficult to discern on IEP but apparent by examination of the autoradiograph below (see also Fig. 6). Rabbit anti-GS Ig was induced in five rabbits by inoculation with purified GS anti-HEA and characteristically contained much more anti-Ig-1 than did rabbit anti-WGS. In fact, antiserum specific for Ig-1 was made by simply adding whole normal GS serum to rabbit anti-GS Ig (Fig. 2, top trough), a procedure which preferentially removed antibodies to Ig-2 and Ig-M (Fig. 2, bottom trough).

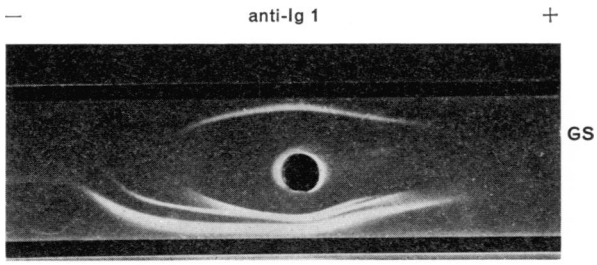

Figure 2. Immunoelectrophoresis of immune GS serum developed with rabbit anti-garter snake Ig (anti-GS Ig) (bottom trough) which precipitated Ig-2, Ig-M and Ig-1. This antiserum was specific for Ig-1 after addition of normal garter snake serum (anti-Ig-1, top trough).

Specific anti-Ig-M was obtained by immunizing rabbits with Pevikon-Sephadex isolated serum Ig-M or whole bile (Portis & Coe, 1975) and absorbing non-specific antibodies with LMW-Ig-G. However, rabbit anti-bile Ig-M (anti-GS Ig-M, Fig. 1, bottom IEP trough) was routinely used as it was easier to absorb; only trace amounts of non-specific (L-chain) antibody was produced by late antisera in contrast to rabbit antibody to serum Ig-M which cross-reacted extensively and non-discriminately with both LMW-Ig. Rabbit antisera against GS serum Ig-M and bile Ig-M each contained antibodies for a non-Ig protein which were removed by absorption with Pevikon block and Sephadex G-200 isolates.

Physical properties of snake Ig

Fig. 3 shows the results from filtration of whole

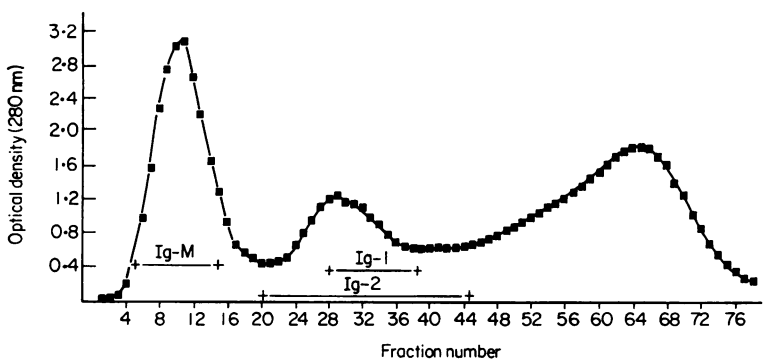

Figure 3. Sephadex G-200 filtration of normal garter snake serum with Ig-M detected only in first (exclusion) peak and Ig-1 and Ig-2 found in the second peak.

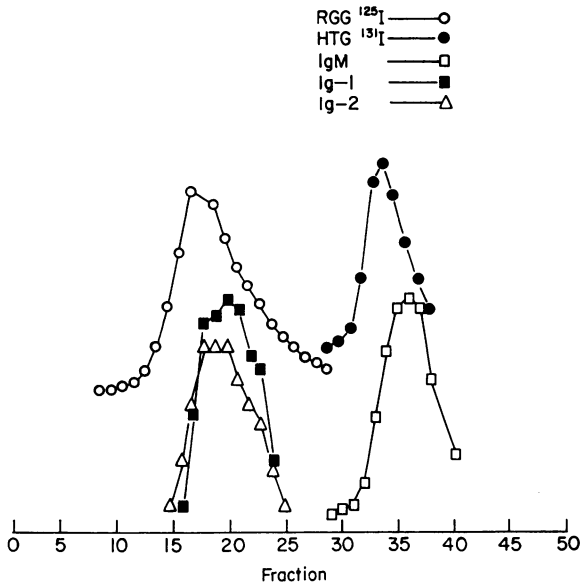

Figure 4. Sucrose density gradient (10–44 per cent, SW50·1, 35,000 r.p.m. ×18 h) of immune snake serum. Fractions were removed from top of gradient. When compared to marker proteins (^{125}I-labelled RGG and ^{131}I-labelled HTG), S values were: Ig-1 ≃9, Ig-2 ≃8·5, Ig-M ≃20. The ordinate represents a linear normalized scale for c.p.m. (RGG and HTG) and for arbitrary standard snake Ig concentration units.

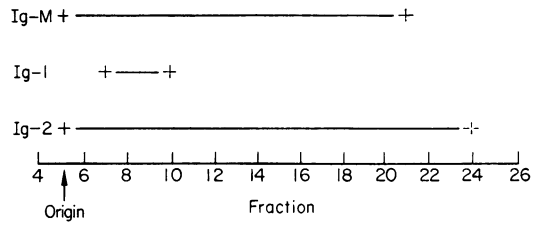

Figure 5. Distribution of snake Ig in fractions from Pevikon block preparative electrophoresis (400 V × 18 h) of normal GS serum. Origin at fraction 5, anode at right. Limited heterogeneity of Ig-1 probably due to small amount present in normal serum.

normal snake serum through Sephadex G-200. HMW-Ig-M was detected in the first (exclusion) peak and LWM-Ig-1 and Ig-2 were found in the second peak. The third peak consisted mainly of albumin. (Three sedimentation peaks ≃20S (not shown), 8 and 4S also were obtained by analytical ultracentrifugation (Fig. 8).) When normal serum was analysed by 10–44 per cent SDG (Fig. 4), HMW-Ig-M sedimented at ≃20S, Ig-1 at ≃9S and Ig-2 slightly slower at ≃8·5S. Although these S values are approximate, Ig-1 characteristically sedimented a little faster than Ig-2 on similar gradients.

Electrophoresis of normal serum on Pevikon (Fig. 5) showed extensive migration of Ig-M and Ig-2 but only limited electrophoretic heterogeneity of Ig-1, probably because of the small concentration in normal serum.

Snake Ig response to purified protein antigens

The formation of Ig-1, Ig-2 or Ig-M antibody was determined by RIEP with sera obtained at various intervals after inoculation of five snakes each with either HEA, HGG or KLH (Table 1).

Thirty-one days after primary inoculation, only five of fifteen snakes had developed antibody and three of these had only Ig-M antibody whereas two snakes also had Ig-2 antibody. Seventeen days later (48 days after primary inoculation and 17 days after booster injection) almost all (13/14) snakes had serum antibody. Most had antibody in all three Ig types although five had only Ig-M and Ig-2 antibody. By day 75, all fifteen snakes examined had antibody in all three Ig classes.

Antibody was detected for prolonged periods in snakes. The response to HGG and KLH was stronger than HEA as shown by more binding on RIEP with early and later sera and good binding of KLH by Ig-1 and Ig-2 in one serum obtained almost three years after inoculation. Although RIEP detected only binding capacity, the snake antibody was shown to be precipitating as detected on agarose slides with double diffusion against specific antigen.

Ig-M antibody disappeared first since it was found in only two of fourteen sera on day 160 and was not detectable in later sera. Next, Ig-2 antibody became less prominent after day 160. Fig. 6 compares sera from the same snake; between day 75 and 186, Ig-M anti-HEA became undetectable and the Ig-2 anti-HEA decreased and was just barely detectable in the Ig-2 line of anti-GS Ig. Late anti-HEA sera (day 273 and 384) contained only Ig-1 anti-HEA (Table 1), and Ig-2 binding was consistently less than Ig-1 with other late sera. Thus, Ig-1, which was the least obvious Ig in normal serum appeared to be the most prominent Ig in the antibody response to antigens in Freund's adjuvant.

Ig-1 also showed the most striking increase when snake sera were tested for changes in concentration

Table 1. Presence of antigen binding by Ig-1, Ig-2 and Ig-M in garter snake sera obtained on various days after immunization with HEA, HGG and KLH

	Day after inoculation									
Antigen	13	31*	48	56*	75	122*	150–160	273	384	992
HEA FCA (0·5 mg)	5 (none)†	5 (none)	1 (none) 2 (Ig-M, Ig-2) 2 (Ig-M, Ig-1, Ig-2)	1 (none) 1 (Ig-M, Ig-2) 3 (Ig-M, Ig-1, Ig-2)	5 (Ig-M, Ig-1, Ig-2)		5 (Ig-1, Ig-2)	3 (Ig-1)	1 (Ig-1)]	
HGG FCA (0·5 mg)	5 (none)	1 (none) 3 (Ig-M) 1 (Ig-M, Ig-2)	1 (Ig-M, Ig-2) 4 (Ig-M, Ig-1, Ig-2)	5 (Ig-M, Ig-1, Ig-2)	5 (Ig-M, Ig-1, Ig-2)		5 (Ig-1, Ig-2)		1 (Ig-1, Ig-2)	
KLH FCA (0·5mg)	5 (none)	4 (none) 1 (Ig-M, Ig-2)	2 (Ig-M, Ig-2) 2 (Ig-M, Ig-1, Ig-2)	5 (Ig-M, Ig-1, Ig-2)	5 (Ig-M, Ig-1, Ig-2)		2 (Ig-M, Ig-1, Ig-2) 2 (Ig-1, Ig-2)	1 (Ig-1, Ig-2)	1 (Ig-1, Ig-2)	1 (Ig-1, Ig-2)
Summary	15 (none)	10 (none) 3 (Ig-M) 2 (Ig-M, Ig-2)	1 (none) 5 (Ig-M, Ig-2) 8 (Ig-M, Ig-1, Ig-2)	1 (none) 1 (Ig-M, Ig-2) 13 (Ig-M, Ig-1, Ig-2)	15 (Ig-M, Ig-1, Ig-2)		12 (Ig-1, Ig-2) 2 (Ig-M, Ig-1, Ig-2)	3 (Ig-1) 1 (Ig-1, Ig-2)	1 (Ig-1) 2 (Ig-1, Ig-2)	1 (Ig-1, Ig-2)

* 0·5 mg antigen IF booster inoculation.
† Numeral before parentheses indicates the number of snakes with no antibody (none) or antibody in either Ig-M, Ig-1, Ig-2.

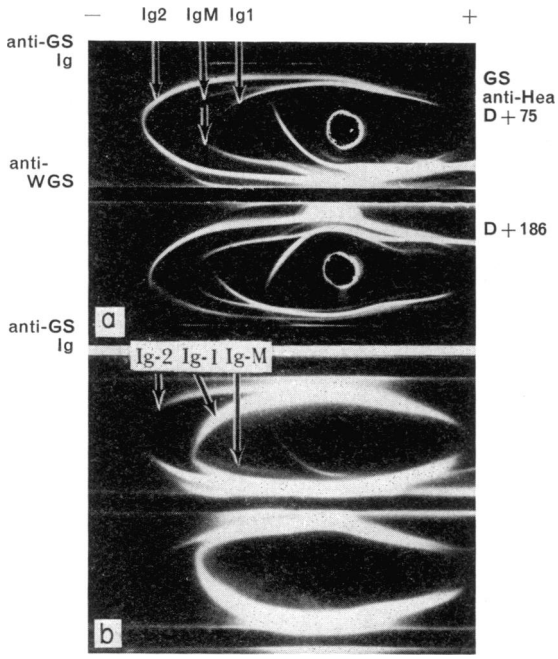

Figure 6. Immunoelectrophoresis slide (a) and respective ^{131}I-labelled HEA autoradiograph (b) of serum obtained from one garter snake 75 days (top IEP pattern) and 186 days (bottom IEP pattern) after primary immunization. Note the prominent binding of ^{131}I-labelled HEA by Ig-1 in both sera and the decrease of Ig-2 and Ig-M binding from days 75–186. The radioactive arc just cathodal to the day 75 well represents ^{131}I binding occasionally found in normal sera and thought to be non-Ig binding.

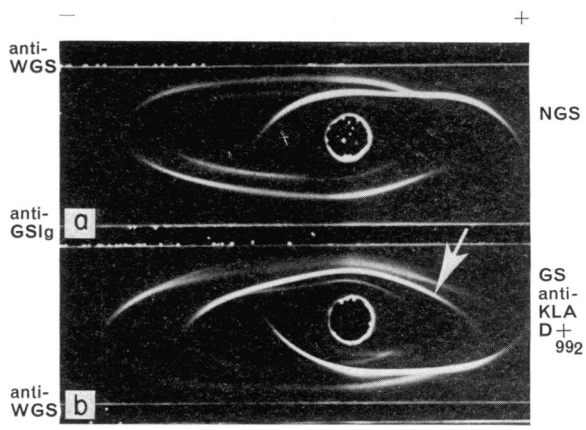

Figure 7. Immunoelectrophoresis of normal GS serum (NGS) (a) and immune GS anti-KLH (day+992) (b). Note that the Ig-1 precipitin line (arrow) is only detected with rabbit anti-GS Ig (middle trough) against immune GS and is not seen in anti-GS Ig reaction with NGS.

of serum Ig after immunization with Freund's adjuvants. Comparison of sera obtained on days 0 and 160 from the same snakes, showed an 8–16-fold increase in Ig-1 and 4-fold increase in Ig-M. Fig. 7 compares the IEP of normal and KLH-immune snake serum. Typically, only two lines (Ig-M and Ig-2) were detected with anti-GS Ig (middle trough) in normal GS (NGS) sera whereas Ig-1 (Fig. 7, arrow) was also seen in Freund's immune serum, even when sera were obtained almost 3 years after inoculation. The increase of $\simeq$8S protein in FCA-immune sera was particularly apparent by examination in the analytical ultracentrifuge; the $\simeq$8S peak was barely detectable in the normal serum (Fig. 8b) but quite apparent in the immune serum (Fig. 8a).

Other immune snake sera were examined for changes in Ig-1 concentration. A serum from one snake immunized 151 days previously with RGG-FIA

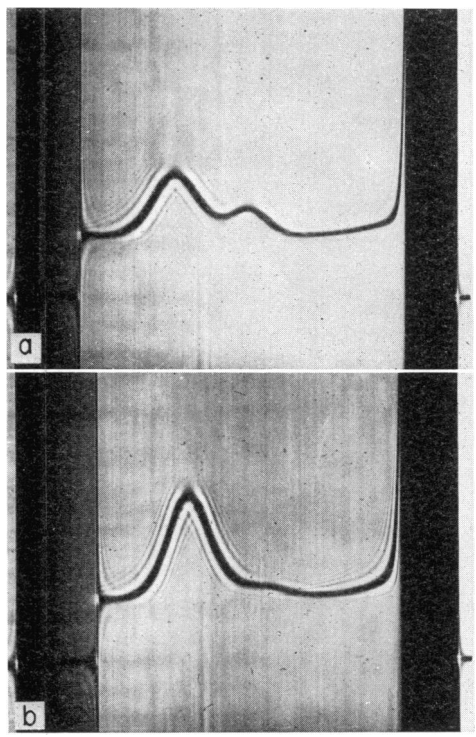

Figure 8. Schliern patterns of normal (b) and FCA-immune (a) sera in the analytical ultracentrifuge 89·7 and 88·01 min respectively after attaining 50,740 r.p.m. Direction of sedimentation left to right. Both sera contained a $\simeq$20S peak (not shown) and a 4S peak (farthest left) however the $\simeq$8S peak is obvious only in the immune serum.

contained eight-fold more Ig-1, and two-fold more Ig-M than normal. On the other hand, sera from snakes containing antibody to western equine encephalitis (WEE) virus (Thomas & Coe, unpublished data), after i.p. injection of an aqueous suspension of infected mouse brain, did not have increased serum Ig-1 although Ig-M was two–four-fold higher than normal.

DISCUSSION

Although reptilian immunity attracted the attention of investigators such as Metchnikoff (1901) many years ago, little is known about snake immunology. Snake antibodies are of some epidemiological interest because of the possible role that snakes may play as a carrier for some arthropod-born viruses such as western equine encephalomyelitis virus (Thomas & Eklund, 1962).

Previous studies have shown that multiple Ig classes exist in reptiles (Ambrosius *et al.*, 1971; Coe, 1972; Leslie & Clem, 1972) so that the presence of one HMW and two LMW Ig in garter snakes is not surprising. More definitive work with isolated Ig will be necessary before these reptilian Ig can be related to each other and to other vertebrate Ig. Preliminary observations (unpublished results) with the rabbit anti-snake Ig used in this study indicate that Ig of other snakes are electrophoretically and antigenically similar with those described in garter snakes.

The present report indicates that garter snakes can produce substantial amounts of antibody for prolonged intervals of time after inoculation with antigen in Freund's adjuvant. The most interesting finding was the peculiar activation of one LMW Ig (Ig-1). Ig-1 was almost undetectable with anti-WGS in normal and in WEE immune serum but it increased up to sixteen-fold in serum concentration after immunization with adjuvants and contained the most antigen-binding capacity for the longest intervals afterwards. It is unlikely that all the increased serum Ig-1 represents antibody to HEA-FCA and part of the increased Ig-1 probably represents a nonspecific stimulation as described in rabbits (Humphrey, 1963). Chickens (Tam & Benedict, 1975) and frogs (Hadji-Azimi, 1973) inoculated with FCA also have increased serum Ig levels and injection of BSA–Freund's adjuvant into cyclostomes (Linna, Finstad, Fichtelius & Good, 1970) induced proliferative responses which correlated directly with the immune responsiveness of the animal. These observations in lower vertebrates are of interest because of the apparent mammalian requirement for thymus-derived cells in an adjuvant response (Allison & Davies, 1971). If lower vertebrate responses are dependent on thymus-like cells, it would follow that snake Ig-1 was particularly dependent and perhaps even the product of a thymus-derived cell. Ig synthesis by a snake thymus-like cell is not unreasonable considering the fact that frog thymus cells have membrane bound Ig detectable by fluorescent microscopy (DuPasquier, Weiss & Loor, 1972; Coe & Portis, unpublished observations).

The mode of immune stimulation achieved with Freund's adjuvant appears to be an unnatural event in garter snakes because it so markedly alters the ratio of Ig concentrations in serum. This situation was advantageous for producing a specific antiserum to Ig-1, i.e. immunizing rabbits with isolated snake anti-HEA (containing relatively large amounts of Ig-1) and absorbing with normal serum (containing low amounts of Ig-1). Thus an antiserum specific for Ig-1 was easily produced as was antiserum to Ig-M by using snake whole bile. On the other hand, specific anti-Ig-2 was not obtained because of the lack of a suitable absorbent (i.e. a preparation not containing Ig-2). Therefore, the existence of a unique class-specific antigen for Ig-2 was not demonstrated and for operational purposes Ig-2 was defined simply as the third precipitin line (after exclusion of Ig-1 and Ig-M) detected on gel diffusion with rabbit anti-snake Ig. The possibility exists that Ig-2 consists only of common Ig antigens such as Fab or L chain-like fragments without unique H-chain determinants. This is unlikely, however, because Ig-2 was similar (although not identical) in size to Ig-1, was a prominent Ig in serum and contained specific antibody independent from and frequently prior to Ig-1 (See Table 1, days 31 and 48). Ig-2 could also possibly represent another L-chain type as κ and λ-like antigenic differences have been detected in the alligator (Soluk, Krauss & Clem, 1970).

Immunological studies of lower vertebrates frequently are hampered by sluggish immune responses and relatively low Ig concentrations. It is apparent from this study that the use of serum from adjuvant-stimulated animals can facilitate the detection of Ig classes which otherwise might be overlooked in normal serum.

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REFERENCES

ACTON R.T., EVANS E.E., WEINHEIMER P.F., NIEDERMEIER W. & BENNETT J.C. (1972) Purification and characterization of two classes of immunoglobulins from the marine toad (*Bufo marinus*). *Biochemistry*, **11**, 2751.

ALLISON A.C. & DAVIES A.J.S. (1971) Requirement of thymus-dependent lymphocytes for potentiation by adjuvants of antibody formation. *Nature (Lond.)*, **233**, 330.

AMBROSIUS H., HEMMERLING J., RICHTER R. & SCHIMKE R. (1971) *Developmental Aspects of Antibody Formation and Structure* (ed. by J. Sterzl and I. Riha), 2nd edn, p. 727. Academic Press, New York.

COE J.E. (1968) The immune response in the hamster. I. Definition of two 7S globulin classes: 7Sγ1 and 7Sγ2. *J. Immunol.* **100**, 507.

COE J.E. (1972) Immune response in the turtle (*Chry-emys picta*). *Immunology*, **23**, 45.

COE J.E. & PEEL L.F. (1970) Antibody production in the bullfrog (*Rana catesbeiana*). *Immunology*, **19**, 539.

CUATRECASAS P. (1970) Protein purification by affinity chromatography. *J. biol. Chem.* **245**, 3059.

DUPASQUIER L., WEISS N. & LOOR F. (1972) Direct evidence for immunoglobulins on the surface of thymus lymphocytes of amphibian larvae. *Europ. J. Immunol.* **2**, 366.

HADJI-AZIMI I. (1973) Serum immunoglobulin content in normal and lymphoid tumor-bearing *Xenopus laevis*. *Cancer Res.* **33**, 1177.

HUMPHREY J.H. (1963) *La Tolérance Acquise et la Tolérance Naturelle à l'Egard de Substances Antigéniques Definies*, p. 401. Centre National de la Recherche Scientifique, Paris, France.

LAURELL C.-B. (1966) Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Analyt. Biochem.* **15**, 45.

LESLIE G.A. & CLEM L.W. (1972) Phylogeny of immunoglobulin structure and function. VI. 17S, 7·5S and 5·7S anti-DNP of the turtle *Pseudamys scripta*. *J. Immunol.* **108**, 1656.

LINNA T.J., FINSTAD J., FICHTELIUS K.E. & GOOD R.A. (1970) Proliferative responses of cyclostomes to stimulation with antigen in adjuvant. *Acta path. microbiol. scand.* **78**, 169.

LYKAKIS J.J. & COX F.E.G. (1968) Immunological responses of the toad *Xenopus laevis* to the antigens of the ciliate *Tetrahymena pyriformis*. *Immunology*, **15**, 429.

MARCHALONIS J. & EDELMAN G.M. (1966) Phylogenetic origins of antibody structure. II. Immunoglobulins in the primary immune response of the bullfrog *Rana catesbeiana*. *J. exp. Med.* **124**, 901.

MARCHALONIS J. & EDELMAN G.M. (1968) Phylogenetic origins of antibody structure. III. Antibodies in the primary immune response of the sea lamprey *Petromyzon marinus*. *J. exp. Med.* **127**, 891.

MCCONAHEY P.J. & DIXON F.J. (1966) A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy*, **29**, 185.

METCHNIKOFF E. (1901) *L'Immunite dans les Maladies Infectieuses*. Masson and Cie, Editeurs, Libraires de L'académie de Médicine, Paris.

PORTIS J. & COE J.E. (1975) IgM the secretory immunoglobulin of reptiles and amphibians. *Nature (Lond.)*, **258**, 547.

SOLUK P.H., KRAUSS J. & CLEM L.W. (1970) The presence of two antigenically distinct light chains (κ and λ?) in alligator immunoglobulins. *Proc. Soc. exp. Biol. (N.Y.)*, **133**, 365.

TAM L.Q. & BENEDICT A.A. (1975) Elevated 7S immunoglobulin and acute phase proteins in adjuvant-injected chickens. *Proc. Soc. exp. Biol. (N.Y.)*, **150**, 340.

THOENES G.H. & HILDEMANN W.H. (1970) Immunological response of Pacific hagfish. II. Serum antibody production to soluble antigen. *Symposium on Developmental Aspects of Antibody Formation and Structure*, p. 711. Czechoslovakia Academy of Sciences, Prague.

THOMAS L.A. & EKLUND C.M. (1962) Overwintering of western equine encephalomyelitis virus in garter snakes experimentally infected by *Culex tarsalis*. *Proc. Soc. exp. Biol. (N.Y.)*, **109**, 421.

WEIGLE W.O. (1964) Immunochemical properties of hemocyanin. *Immunochemistry*, **1**, 295.