PHYLOGENETIC ORIGINS OF ANTIBODY STRUCTURE

II. Immunoglobulins in the Primary Immune Response of the Bullfrog, Rana catesbiana*

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A previous study of elasmobranch antibodies has established that the immunoglobulins of these lower vertebrates have a molecular structure similar to that of immunoglobulins from higher vertebrates (1). In contrast to the higher vertebrates, however, the elasmobranchs so far studied (1, 2) appear to have only one class of immunoglobulins. This class corresponds most closely in its characteristics to the γM-immunoglobulins. Recent studies (3–5) have indicated that anuran amphibians produce antibodies with different ultracentrifugal and electrophoretic properties. The present investigation was designed to determine whether such antibodies represent different classes of immunoglobulins.

We provide evidence that, in response to a single injection of bacteriophage, the bullfrog *Rana catesbiana* produces two classes of antibodies. The first class to appear corresponds to the γM-immunoglobulins of higher forms; subsequently antibodies appear which resemble the γG-immunoglobulins. These results, taken together with earlier work (1, 6), suggest that the genes for heavy chains of γG-type (γ-chains) arose at or prior to the phylogenetic level of anuran amphibians.

Materials and Methods

Immunization.—Each of eight bullfrogs of both sexes, average weight 120 g, was given a single subcutaneous injection of 0.1 mg of bacteriophage f2 (7) in 0.1 ml of frog Ringer's solution. The animals were bled by cardiac puncture at periods from 15 to 90 days after injection of the antigen. The animals were maintained at 25°C during the course of the experiment. Uninjected animals of both sexes were used in the preparation of normal immunoglobulins.

Assay of Antibody Activity.—Sera and serum fractions were titrated for phage-neutralizing activity according to the procedure described by Adams (8). The potency of the antisera and immunoglobulin fractions is expressed in terms of the rate constant for phage inactivation:

$$k = -\frac{\ln (p/p_0)}{c\Delta t}$$

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where p, number of phage plaques at time t; p_0, number of phage plaques at $t = t_0$; c, protein concentration expressed in mg ml^{-1}; and Δt, time in minutes ($t - t_0$).

Where kinetic curves are not given, k is calculated at $\Delta t = 10$ min because inactivation by both immunoglobulin fractions followed apparent first order kinetics during that interval. Experiments performed on the concentration dependence of k showed that apparent first order kinetics were obeyed. Studies on inactivation of antibodies were carried out by performing the usual titration procedure in the presence of 0.1 M 2-mercaptoethanol.

Preparation of Immunoglobulins.—Immunoglobulin fractions were prepared by zone electrophoresis on starch (9) and by gel filtration on Sephadex G-200 (Pharmacia, Uppsala, Sweden) using conditions reported previously (1). Protein concentrations were determined by the modified Folin-Ciocalteu method (10). In addition, the absorbancy at 280 mμ was measured; for comparison $E^{1\%}_{1cm}$ was assumed to be 14.

Reduction and Alkylation and Preparation of Polypeptide Chains.—The details of these procedures have been previously reported (1, 11). In some cases, dithiothreitol (12) was used as the reducing agent.

Immunoelectrophoresis and Double Diffusion in Agar.—These procedures and the immunization schedule for rabbits have been described (13).

Analytical Ultracentrifugation; Ultracentrifugation in Sucrose Density Gradients.—Analytical ultracentrifugation was performed with a Spinco model E ultracentrifuge equipped with automatic temperature control and phaseplate schlieren and interference optics. Molecular weights of the polypeptide chains dissolved in 1 M propionic acid containing 0.5% sucrose were determined according to the high speed equilibrium method of Yphantis (14). Partial specific volumes ($\bar{V}$) were calculated from the amino acid and carbohydrate compositions assuming that the heavy chains contained all of the carbohydrate. Ultracentrifugation in sucrose density gradients was done in a Spinco model L preparative ultracentrifuge using the SW 39 swinging bucket rotor. The procedure has been described before (15).

Starch Gel Electrophoresis in Urea.—The method has been described in previous reports (11). Human γG-immunoglobulin (Cohn fraction II, Lederle Laboratories, Pearl River, New York) and γM-immunoglobulin (prepared by gel filtration on Sephadex G-200 from Cohn fraction III, Pentex Laboratories, Inc., Kankakee, Illinois) were used as standards for comparison in the gel electrophoretic experiments.

Amino Acid Analysis.—This was carried out on duplicate samples according to Spackman, Stein, and Moore (16) using the automatic amino acid analyzer (Beckman model 120C). Recoveries of tyrosine and methionine were more variable than those of the other amino acids, probably because of oxidative degradation during hydrolysis.

Carbohydrate Determination.—Carbohydrates were determined using the anthrone reaction (17) as employed by Müller-Eberhard and Kunkel (18).

RESULTS

Because only small amounts of blood could be obtained from individual frogs and the serum protein concentration was low (approximately 2%), sera from five immunized animals were pooled for zone electrophoresis on starch (Fig. 1). Relatively large amounts of components with low electrophoretic mobilities were observed. Two partially resolved peaks of phage-neutralizing activity were present in this region. Both active fractions were pooled, concentrated by ultrafiltration, and subjected to gel filtration on Sephadex G-200 (Fig. 2). Three components were resolved. Phage-inactivating activity was associated with the first two peaks which correspond in position to macroglobulin (peak I) and 7S

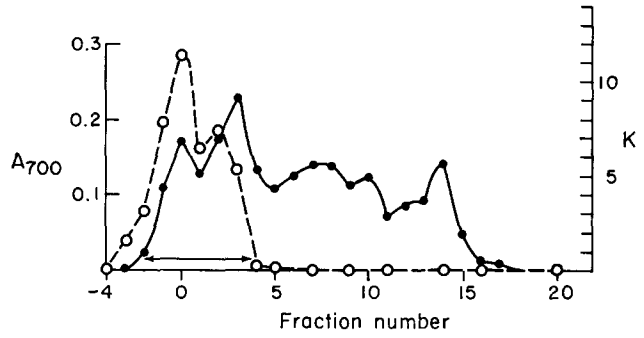


FIG. 1. Fractionation of immune bullfrog serum by zone electrophoresis on starch.
A_{700} (●——●) absorbancy of the Folin reaction at 700 mμ; k (○------○) bacteriophage neutralization constant expressed in mg^{-1} ml min^{-1} (see Materials and Methods for definition). Anode is at right. ○, origin; ↔ fractions pooled for gel filtration.

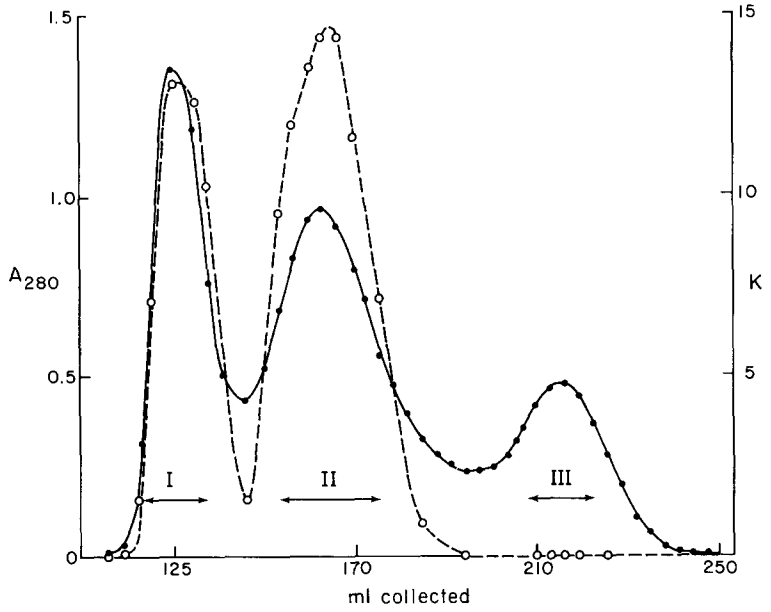
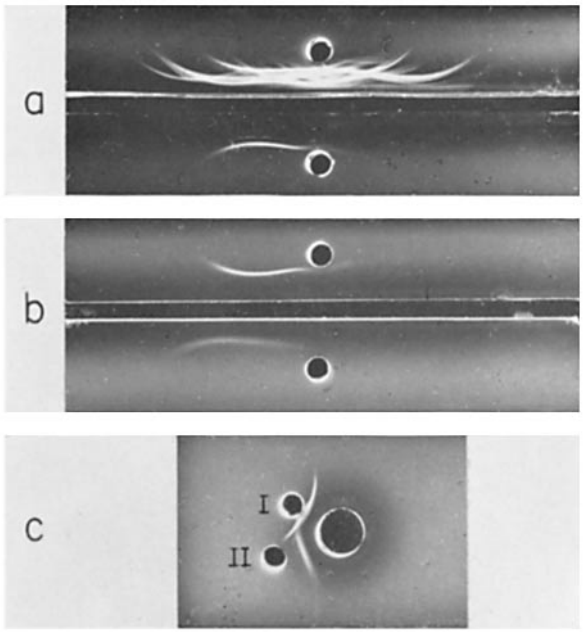


FIG. 2. Gel filtration on Sephadex G-200 of electrophoretic fractions containing immunoglobulins (see Fig. 1).
A_{280} (●——●) absorbancy at 280 mμ; k (○------○) bacteriophage neutralization constant expressed in mg^{-1} ml min^{-1}. I, II, III—fractions pooled for further analysis.

immunoglobulin (peak II). The third peak was pigmented and appeared to be hemoglobin.

An immunoelectrophoretic comparison of whole serum and the two active immunoglobulin fractions is shown in Figs. 3 *a* and 3 *b*. The mobility of material

from peak I was slightly greater than that of the material from peak II. Double diffusion in agar was carried out (Fig. 3 *c*) with rabbit antiserum to material from peak I (macroglobulin). The precipitin arcs showed a reaction of partial identity; material from peak II was antigenically deficient with respect to that from peak I.



FIGS. 3 *a* to 3 *c*. Immunological comparisons of bullfrog serum and immunoglobulins Fig. 3 *a*. Immunoelectrophoresis of whole serum (upper well) and fraction I from gel filtration (lower well). Anode is at right. Antiserum, rabbit antiserum to whole bullfrog serum. Fig. 3 *b*. Immunoelectrophoresis of gel filtration fraction I (upper well) and fraction II (lower well). Fig. 3 *c*. Immune diffusion of immunoglobulins. Antiserum, rabbit antiserum to bullfrog fraction I. I, fraction I from gel filtration; II, fraction II from gel filtration.

An ultracentrifugal analysis of the immunoglobulin fractions showed that peak I material sedimented with $s^{\circ}_{20,w} = 18.0$S, and peak II material was found to have $s^{\circ}_{20,w} = 6.7$S. In the presence of 0.13 M 2-mercaptoethanol the macroglobulin dissociated into components with sedimentation coefficients of approximately 7S. The material from peak II showed diffuse lighter material as well as aggregates after treatment with 2-mercaptoethanol.

Kinetic studies on the 18S and 6.7S immunoglobulin fractions are shown in Fig. 4. The plot for the 6.7S fraction was linear with $k = 20$ mg^{-1} ml min^{-1}. The plot obtained with the 18S immunoglobulin showed curvature after the first 15 min. In the linear region, the macroglobulin plot had $k = 5$ mg^{-1} ml min^{-1}. In-

asmuch as the k values were calculated for total immunoglobulin and the percentage of antibody in each fraction was unknown, the efficiencies of the two kinds of antibody could not be directly compared.

In higher forms, it has been established that injection with a single dose of bacteriophage leads to production of γM-antibodies followed later by production of γG-antibodies (19). A similar sequence of events was observed in the bullfrog. Sucrose gradient patterns of individual sera at different times of immunization are presented in Figs. 5 *a* to 5 *d*. Phage neutralizing activity was initially located only in the macroglobulin peak (Figure 5 *a*). Activity in the 7S peak gradually increased, however, so that by day 90 it accounted for over 90% of the observed neutralization. Two animals were bled at each time point; the patterns of the duplicates corresponded closely with those shown in Figs. 5 *a* to 5 *d*.

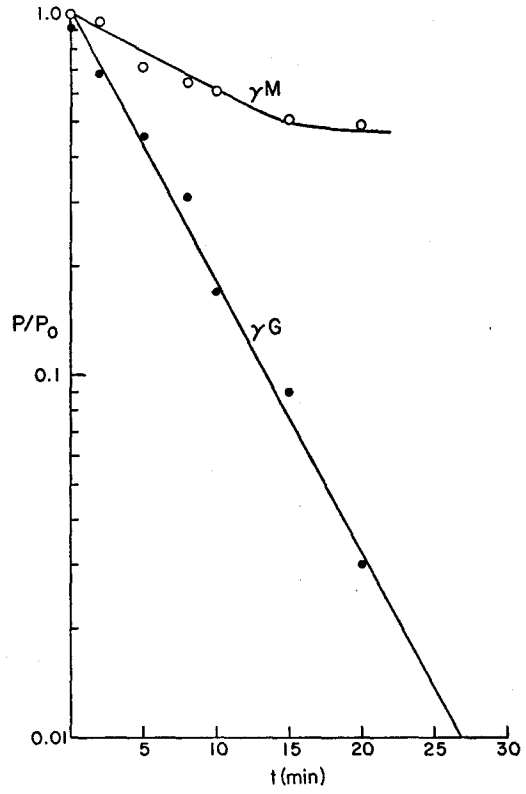
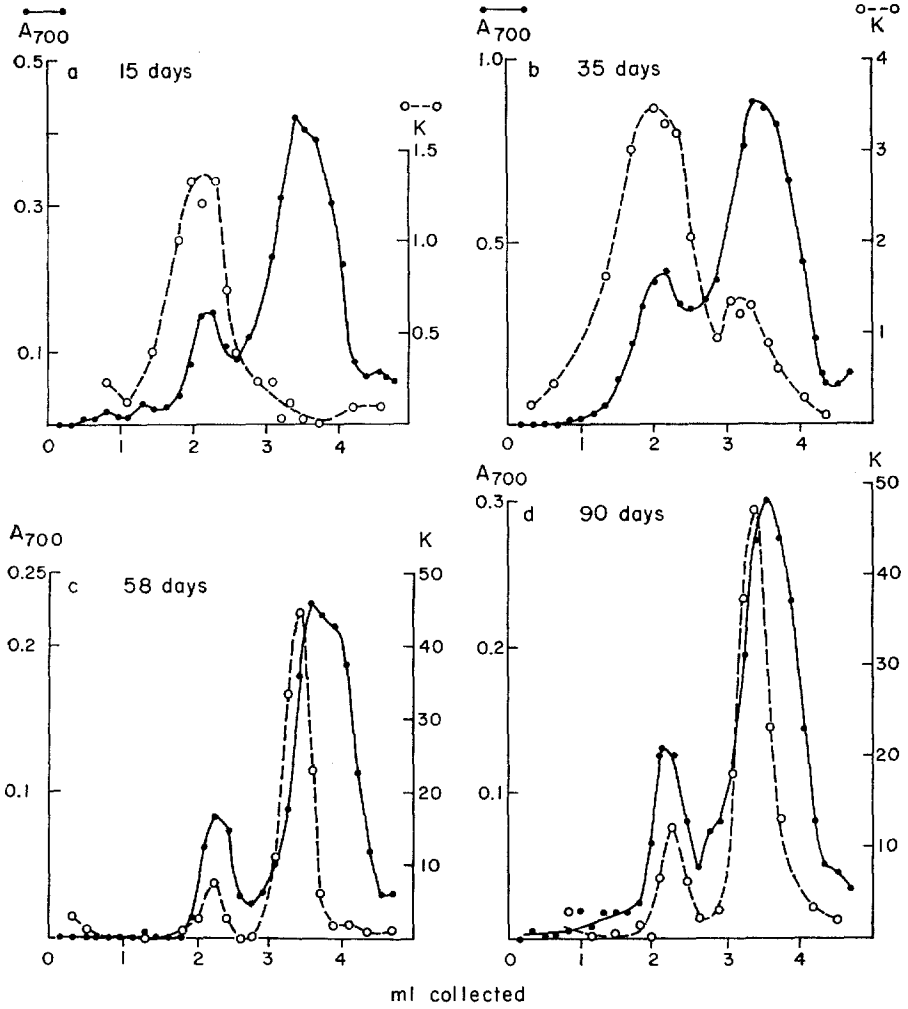


FIG. 4. Kinetics of bacteriophage neutralization by bullfrog γM- and γG-immunoglobulins. ○, γM-immunoglobulin; ●, γG-immunoglobulin; and p/p_0, ratio of plaques remaining at time t to original number of plaques.



FIGS. 5 *a* to 5 *d*. Sequence of appearance of immunoglobulin activity in immunized bullfrogs as shown by ultracentrifugation on linear sucrose gradients.

A_{700} (●——●) absorbancy of the Folin reaction at 700 mμ; *k* (○——○) bacteriophage neutralization constant expressed in mg^{-1} ml min^{-1}. Sedimentation proceeded from right to left: Fig. 5 *a*, 15 days after immunization; Fig. 5 *b*, 35 days after immunization; Fig. 5 *c*, 58 days after immunization; and Fig. 5 *d*, 90 days after immunization.

The values for the inactivation constants at different times are presented in Table I. Both immunoglobulins showed an increase in the value of *k* as time after immunization increased. Also presented in this table are the effects on *k* after treatment of the 6.7S and 18S immunoglobulins with 0.1 M 2-mercapto-

ethanol. The activity of both fractions was diminished considerably by this reagent. In higher forms, only the γM-immunoglobulins are affected in this manner (20) and thus one may conclude that the 6.7S immunoglobulin of the frog differs in this aspect of its structure from γG-immunoglobulins of mammals. The inactivation of frog 6.7S immunoglobulin is probably correlated with the dissociation and aggregation observed in the ultracentrifugal experiments described above.

The 6.7S and 18S immunoglobulins were degraded into their constituent polypeptide chains by reduction and alkylation followed by dissociation in appropriate solvents. Starch gel electrophoresis in 8 M urea (Figs. 6 *a* and 6 *b*) showed the presence of light and heavy polypeptide chains. The two immuno-

TABLE I

Neutralization of Bacteriophage f2 by Bullfrog Antibodies

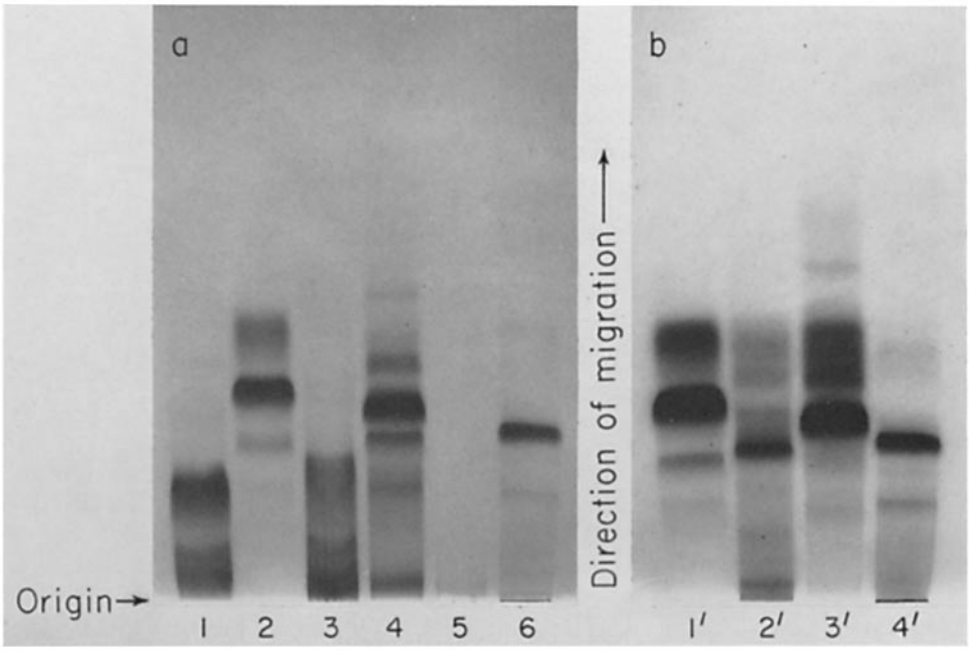
Preparation	After f2 injection	k^* Before mercaptoethanol	k^* After mercaptoethanol
	days		
Normal serum 7	0	0.0	—
Normal serum 8	0	0.0	—
γM-immunoglobulin	35	2.7	0.6
γG-immunoglobulin	35	0.6	0.2
γM-immunoglobulin	58	2.2	0.3
γG-immunoglobulin	58	21.8	3.3
γM-immunoglobulin	90	12.6	6.0
γG-immunoglobulin	90	51.4	5.3

* See materials and methods for calculation of *k*.

globulins had light chains with mobilities similar to those of human immunoglobulins. A definite banding was observed in the bullfrog light chain region, however. The heavy polypeptide chains of the bullfrog 18S immunoglobulin had the same mobility as μ-chains of human γM-immunoglobulin (compare origins 2′ and 4′). Heavy chains of the bullfrog 6.7S immunoglobulin had mobilities greater than μ-chains but slightly lower than those of human γ-chains (compare origins 2 and 4 and 1′ and 3′).

Light and heavy polypeptide chains were fractionated from reduced alkylated 18S and 6.7S immunoglobulins by gel filtration on Sephadex G-100 in 1 N propionic acid, or in 1 N propionic acid, 6 M urea (21). The maximal yield of light chains was 28%, in good agreement with the yields found for mammalian immunoglobulins. The macroglobulin light chain ($\bar{V}$ = 0.726) and the 6.7S immunoglobulin light chain ($\bar{V}$ = 0.724) had molecular weights of 20,000 ±

1000 and 22,000 ± 500, respectively. Heavy chains from the macroglobulin had a molecular weight of 72,100 ± 1600 (based on V = 0.722). Heavy chains from the 6.7S immunoglobulin had a molecular weight of 53,600 ± 1600 (V = 0.729). These values are similar to those obtained for polypeptide chains from γM- and γG-immunoglobulins of rabbit and man (22, 23, 24).



FIGS. 6 *a* and 6 *b*. Starch gel electrophoretic comparisons of untreated and reduced alkylated immunoglobulins of bullfrog and man. All gels were prepared in 8 M urea, formate buffer. Reduced samples were treated with 2-mercaptoethanol in 8 M urea.

Fig. 6 *a*., origins: (*1*) human γG-immunoglobulin; (*2*) reduced alkylated human γG-immunoglobulin, (*3*) bullfrog γG-immunoglobulin, (*4*) reduced alkylated bullfrog γG-immunoglobulin, (*5*) bullfrog γM-immunoglobulin; and (*6*) reduced alkylated bullfrog γM-immunoglobulin. Fig. 6 *b*., origins: (*1′*) reduced alkylated human γG-immunoglobulin; (*2′*) reduced alkylated human γM-immunoglobulin; (*3′*) reduced alkylated bullfrog γG-immunoglobulin; and (*4′*) reduced alkylated bullfrog γM-immunoglobulin.

Amino acid analyses of the immunoglobulins and their chains are given in Table II. The data are consistent with the immunological and electrophoretic results and show that the bullfrog μ-chain differs from the γ-chain whereas the light chains of both classes are similar. The amino acid composition of bullfrog immunoglobulins resembles that of immunoglobulins of higher forms. Acidic residues comprise approximately 20% of the amino acids, basic residues

about 10%, and hydroxy amino acids 15 to 20%. The polypeptide chains of bullfrog immunoglobulins contain a higher percentage of acidic amino acids than do their human counterparts.

Carbohydrate determinations by the anthrone method were carried out on the γM- and γG-immunoglobulins of the bullfrog. The γM-immunoglobulin

TABLE II

Amino Acid Composition of Bullfrog Immunoglobulins and Their Polypeptide Chains

Amino acid*	Bullfrog						Human chains		
	Immunoglobulins		Chains				μ‡	γ§	Light§
	γM	γG	μ	γ	Light (γM)	Light (γG)			
Lys	8.5	8.4	8.8	8.3	7.5	7.2	6.2	7.4	6.2
His	1.8	1.8	2.0	1.6	1.7	1.9	2.5	2.6	1.8
Arg	3.7	4.9	3.0	4.3	4.6	5.1	6.5	3.8	4.6
Asp	12.9	11.2	14.2	12.4	9.7	10.4	9.0	7.6	7.3
Thr	9.1	9.8	8.6	8.2	9.6	9.9	8.8	6.8	7.4
Ser	8.9	8.7	8.8	7.8	9.2	9.3	8.4	8.6	10.3
Glu	12.5	12.2	11.1	12.0	13.5	13.3	12.2	10.1	13.0
Pro	5.4	5.7	5.2	5.5	4.8	4.7	6.5	6.4	5.1
Gly	3.8	4.1	3.5	5.1	4.9	4.6	3.8	3.2	3.3
Ala	3.5	3.0	3.8	3.2	3.3	3.0	4.4	2.7	4.2
Val	6.8	6.9	7.0	6.9	6.4	6.0	8.4	8.2	6.3
Met	0.7	0.5	1.0	1.3	1.3	1.9	1.5	1.0	0.4
Ileu	6.6	6.3	6.9	6.2	5.4	5.4	3.4	1.9	2.7
Leu	6.7	6.7	6.6	7.3	6.0	5.9	8.1	7.1	7.3
Tyr	4.3	5.8	4.8	6.1	7.0	6.7	4.6	5.5	6.2
Phe	5.1	4.2	4.8	4.1	5.0	4.6	5.4	3.8	4.3
Total	100.3	100.2	100.1	100.3	99.9	99.9	99.7	86.7	90.4

* Amino acid composition is expressed as g/100 g of carbohydrate-free protein. Values for cysteine and tryptophan were not included in the total or in the calculation of percentages.

‡ Calculated from Chaplin et al. (25).

§ Data of Crumpton and Wilkinson (26), which include cysteine and tryptophan.

contained 10.8 ± 2.6% carbohydrate and the γG-immunoglobulin contained 2.1 ± 0.1% carbohydrate.

DISCUSSION

The different immunoglobulin classes in mammals are characterized by the presence of class distinctive heavy chains (27, 28). As demonstrated in the present study, a similar situation obtains for γG- and γM-immunoglobulins of

the anuran amphibian, *Rana catesbiana*. The sequence of response to a single injection of phage antigen also resembles that found in higher forms. Phage-neutralizing activity is first associated with γM-immunoglobulins and in later stages of immunization is found preponderantly in the γG-immunoglobulins.

In mammalian sera, γM-immunoglobulin comprises 5 to 10% of the total immunoglobulins (29). In contrast, the total bullfrog immunoglobulins contain approximately 40% γM-immunoglobulin. Relatively high amounts of macroglobulin have been previously observed in frog sera (5) as well as in the sera of elasmobranchs (1) and reptiles (20).

Experiments on the subunit structure of the frog immunoglobulins showed a close similarity to corresponding classes in mammals. Upon starch gel electrophoresis the light chains of both immunoglobulins had a mobility range very similar to that of human light chains. The bullfrog light chains show a definite banding at acid pH in urea, however. This suggests that there may be a different distribution of net charge in the light chains of the two species.

As in other species, the heavy chains of γG- and γM-immunoglobulins penetrate the gel to different extents. The macroglobulin heavy chain had the same mobility as human μ-chains, whereas the 6.7S immunoglobulin heavy chain moved slightly behind the human γ-chains. It is possible, therefore, that the 6.7S immunoglobulin might correspond to γA-immunoglobulins, the heavy chains (α-chains) of which have intermediate mobilities on the gel. This interpretation is made unlikely, however, by the finding that the carbohydrate content of the frog 6.7S immunoglobulin is in the range observed for γG-immunoglobulins of other species.

The general resemblance of the bullfrog macroglobulin and 6.7S immunoglobulin to human γM- and γG-immunoglobulins (7) respectively is further suggested by: (*a*) The behavior on immunoelectrophoresis; the 6.7S immunoglobulin has a lower mobility than the macroglobulin. (*b*) The antigenic relationships; although immunologically related, the 6.7S and 18S immunoglobulins are not identical. (*c*) The carbohydrate content; the macroglobulin contains 10.8% carbohydrate whereas the 6.7S immunoglobulins contain 2.1%. (*d*) The molecular weights of subunits; the macroglobulin heavy chain has a molecular weight of 72,100 whereas that of the 6.7S immunoglobulins has a molecular weight of 53,600 (24). Light chains of both classes have molecular weights of approximately 20,000 (24).

The yields obtained after isolation of the chains by gel filtration and the molecular weight measurements suggest that the chain structure of frog immunoglobulins also closely resembles that found in higher animals. The γG-immunoglobulin appears to have a four-chain structure, whereas that of the γM-immunoglobulin consists probably of five four-chain units linked by disulfide bonds.

Both classes of bullfrog antibodies are susceptible to inactivation by 2-mer-

captoethanol. In the case of the macroglobulin, the inactivation is correlated with dissociation to 7S-8S subunits as in higher forms (30). Loss of activity of the γG-antibody is probably related to partial dissociation and aggregation. The partial dissociation of the reduced γG-immunoglobulin in the absence of denaturing solvents resembles the dissociation observed in avian (31, 32) and reptilian (20) immunoglobulins.

The results discussed above raise an interesting evolutionary possibility. Since elasmobranchs possess only a single class of immunoglobulin (apparently containing heavy chains of the μ-type) and bullfrogs possess immunoglobulins with either μ- or γ-chains, it appears that the genes which code for γ-chains emerged at or prior to the level of anuran amphibians. This is consistent with the previous suggestion (1) that the order of appearance of different classes of immunoglobulins in the immune response is the same as the order of emergence of their heavy polypeptide chains in evolution.

SUMMARY

The anuran amphibian, *Rana catesbiana*, has been found to possess at least two kinds of immunoglobulins corresponding to γG- and γM-classes. These classes have the same chain structures as those of their counterparts in higher animal species. Light chains of both immunoglobulins had molecular weights of 20,000. Heavy chains of the γM-class had molecular weights of 72,100; those of the γG-class had molecular weights of 53,600. The carbohydrate content of the γG-immunoglobulin was 2.1%, and that of the γM-protein was 10.8%. The amino acid compositions of the immunoglobulins were generally similar to those of mammalian immunoglobulins.

After a single injection of phage antigen (f2), the order of appearance of phage-neutralizing activity in the frog immunoglobulin classes was (*a*) γM-antibodies, and (*b*) γG-antibodies.

The results of this and previous studies suggest that the γG-immunoglobulins emerged at some point in evolution between the elasmobranchs and the anuran amphibians.

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