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Functional Egg Immunoglobulins in the snake *Elaphe guttata*

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Adaptive immunity, when vertically transmitted from mother to offspring, creates an essential advantage for the fitness of a species. The transfer of maternal antibodies provides a considerable degree of protection to the offspring from birth on, especially against virulent pathogens (Powell, 1987). Although it has been known since long time that birds are able to accumulate a considerable amount of antibodies in the egg yolk (Klemperer, 1883), and a deposition of immunoglobulins in eggs is even documented in fish (e.g. Hayman and Lobb, 1993), only very scarce information is available about this phenomenon in reptiles. Hassl and Hassl (1986) demonstrated that immunoglobulins are a component of the egg content of colubrid snakes, but a detailed characterisation of the egg antibodies is still lacking. The aims of this study were the axiomatic demonstration of a consistent transmission of specific immunoglobulins via snake eggs and a biochemical characterisation of these immunoglobulins.

Ten unhatched, fertilised eggs of two captive borne, four years old sibling females of *Elaphe guttata* were taken in 1985 for antibody isolation. Six month before the egg laying both snakes were immunised with highly pure bovine serum albumin (BSA, Merck Darmstadt, FRG) by the conventional adjuvant method aiming to produce a massive secondary immune answer.

The egg contents were mixed and pooled, the volume was determined, and the fraction containing immunoglobulins was isolated in triplicate as described by Hassl and Hassl (1986). In short, according to the well-founded recipes for chicken egg antibody isolation (Polson et al., 1985; Polson, 1990), the snake egg immunoglobulins were isolated and purified by precipitation with 3.5%, 12% PEG 6000 (Merck), and ethanol. The resulting solutions of soluble proteins were quantitatively measured for their protein content in 280 nm light using BSA as standard. These fractions were frozen at -70°C for later characterisation.

A further purification of the egg immunoglobulins was attempted by a binding of the Fc-parts to Protein A of *Staphylococcus aureus* applying an affinity chromatography technique with Protein A-Sepharose CL-4B, accurately as described by the reagent manufacturer (Pharmacia GesmbH, Vienna).

A solution of pure, specific egg antibodies was created by specific binding of the immunoglobulins to the antigen (BSA) coupled to an affinity chromatography matrix, namely to Blue Sepharose 6 Fast Flow (Pharmacia GesmbH). Coupling of antigen to the matrix was strictly performed according to the manufacturer's advice, the affinity chromatography in the way of a fast performance liquid chromatography (FPLC) was performed according to the proposal of the equipment manufacturer (Pharmacia GesmbH). In short, egg antibodies were bound to the immobilised antigen under mild, physiological conditions (0.15 M phosphate buffer pH 7.4 + 0.1% Tween 20) for 2 h, unbound substances were washed away, and the antibodies were eluted with 0.1 M glycine-HCl pH 4.0, and the solution was neutralised with

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1 M Tris-HCl. The protein-containing fractions were recognised in 280 nm light and collected. The corresponding fractions of 12 isolation procedures were pooled, and the pool was frozen for further characterisation.

The antibody fractions were primarily characterised by a gel filtration in a Superose 6 HR 10/30 column (Pharmacia GesmbH). This technique determines the molecular mass (mm) of native proteins in the range between 10 and 1000 kD and it desalts the solution. Secondly, the antibodies were characterised by determining the isoelectric points in an isoelectric focussing technique (pH 3-10), using commercially available standard proteins, all done according to the manufacturer's recommendations (Pharmacia GesmbH). Moreover, all fractions were tested for their antigen-binding capacity in an agar gel immunoprecipitation test as described by Hassl et al. (1987) for chicken egg yolk antibodies.

Specific egg antibodies were isolated in substantial amounts, they are thus an essential component of the content of eggs laid by previously immunised corn snakes. About 3.6% of the soluble egg proteins (average 6.3 g/egg) were immunoglobulins, and about 0.3% of this fraction were specific antibodies. Immunoglobulin isolation by Protein A-chromatography was unproductive: *Elaphe* egg immunoglobulins did not bind to Protein A in substantial amounts, but, a small portion of all antibodies, characterised by a singular isoelectric point of pH 5.75, seemed to bind. The mm of the native egg antibody was about 175 kD. This finding proved that the isolated antibody was a member of the Y class. According to the results of the gel filtration (fig. 1) the most plausible mm of the native Fab'-part was about 38 kD, and the one of the Fc part was about 57 kD. The isoelectric points of the *Elaphe* egg immunoglobulins and their fragments and domains were at pH 5.4; 5.56; 5.75; 7.07; 7.4; 8.19; and 8.34.

Although the transmission of immunoglobulins from mothers to their offspring is a well known phenomenon in vertebrate phylogenesis, and is even known to occur in fish (e.g. Hayman and Lobb, 1993), a convincing prove of a vertical transmission of specific antibodies in reptiles has never been given. This may be partially due to the fact that (1) reptiles are not bred on a business-related basis for immunological studies, and (2) reptiles are less susceptible to immunisation than birds. Nevertheless, understanding the phenomenon of antibody transmission via the egg has significant impact on the explanations of some unique immunological observations (e.g. Zapata et al., 1992; Uller et al., 2003).

In all vertebrates increased titres of immunoglobulins in the serum are regularly found after contact with substances which effect as antigen. Moreover, some antibody classes are deposed in eggs. In birds, they are found in the yolk, and they begin to be absorbed in the last stages of embrionic development until shortly after hatch (Powell, 1987).

The appearance of a vertical antibody transmission in reptiles has long been supposed; nevertheless, an axiomatic proof can only be produced by the demonstration of a specific immunreaction between reptile egg antibody and an antigen (Hassl and Hassl, 1986). We choose bovine serum albumin, mm 67 kD, as antigen for snake immunisation, because serum of reptiles does not naturally react with this protein (deSmet, 1978). We thus avoided any problems with naturally occurring antibodies. Purified chicken yolk antibodies of the common Y class were used as comparative material, whereas snake serum antibody production characteristics were not studied simultaneously due to poor precognition and to ethical considerations.

Immunoprecipitation assay and the isolation of a specific antibody fraction by affinity chromatography with immobilised antigen coercively proved the existence of specific antibodies in eggs of corn snakes. According to deSmet (1976) chicken serum has a protein concentration of 42 mg/ml, and of the proteins about 25 mg are globulins, the albumin/globulin ratio is 0.5; one ml of *Elaphe* serum contained about

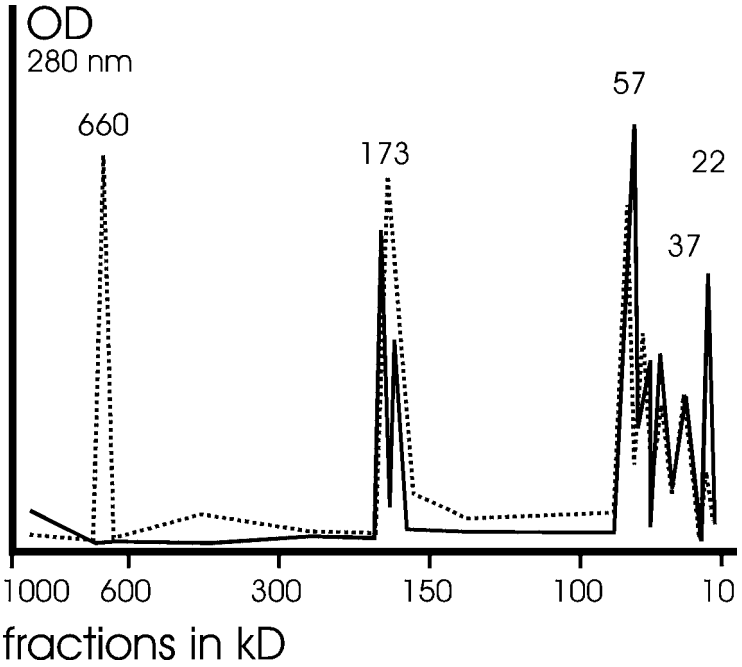

Figure 1. Graph of absorbance at 280 nm of the fractions, sorted according to their molecular mass in kD, of a gel filtration on a Sepharose 6 HR 10/30 column of the purified *Elaphe* egg immunoglobulins (dotted line), and these immunoglobulins after an antigen-antibody reaction affinity chromatography purification step (full line). Numbers indicate approximate molecular masses of the proteins.

47.5 mg proteins and about 40 mg globulins, the albumin/globulin ratio was 0.1. Although the yield of any antibody extraction is strongly dependent on the efficiency of the isolation procedure, and optimised procedures are not available for reptile immunoglobulin purification, as much as 36 mg immunoglobulins per ml egg slurry could be isolated. This amount is almost equal to the quantity of immunoglobulins in the serum of *Elaphe* snakes (40 mg). In chicken, the situation is more complicated due to more antibody classes present, nevertheless, avian IgY serum concentration is about 5-10 mg/ml, and yolk IgY concentration is also about 10 mg/ml (Jensenius et al., 1981). Thus, chicken egg yolk as well as snake eggs contain roughly as much antibodies as the serum, respectively.

Elaphe snakes are assumed to produce two immunoglobulin classes, an early appearing, 19 S, ancestral IgM antibody, and an IgY with a sedimentation constant of 7 and a mm of about 150-180 kD (ElRidi et al., 1991; Warr et al., 1995). The IgY is the typical low-molecular-weight serum antibody in birds, reptiles, and amphibians (Warr et al., 1995), and the only one of this type in most reptiles. The *Elaphe* egg immunoglobulin has a mm of about 170 kD (fig. 1, and Hassl and Hassl, 1986), does not fix mammalian complement (data not shown), and binds specifically to the antigen in an immunoprecipitation assay. These features prove the immunoglobulin to be a not-truncated antibody of the Y class. The mm of chicken IgY fragments are 44 kD for the Fab'-parts, 52 kD for the Fc-part, and 67 kD for a solitaire heavy chain (Akita and Nakai, 1993). Snake serum IgY consists of two heavy chains with 63 and 50 kD, and two light chains with 23 and 20 kD (ElRidi et al., 1991). We detected peaks at 57 kD, the heavy chains, at 37 kD, most probably the Fab'-parts, and at 22 kD, the light chains. But, these findings indicate that the purification conditions we applied were too harsh, as they led to a partial disintegration of the antibody. Some unusual

properties of chicken IgY associated with high salt concentrations have been reported, they include enhanced precipitation of Fc crystals and trimer formation with mm of 550-590 kD (Akita and Nakai, 1993), and fragment agglomeration with mm of 440, 200 and 165 kD (Yazawa et al., 1991). The high molecular weight peak in the purified fraction of the snake egg antibody shown in figure 1 may be an expression of such a polymer formation.

The binding capacity of IgY to Protein A is a topic of contradictory discussion. Similar to the situation in chicken (Barkas and Watson, 1979), we found a small portion of the pure immunoglobulin fraction to react with this strong mammalian antibody captor. The results of the isoelectric focussing experiment demonstrate the existence of a distinct Protein A-binding IgY-antibody fraction. Taken together, there seem to exist at least two isotypes of the egg antibody in corn snakes. As an alternative hypothesis we have to postulate a different antibody formation or transmission competence between corn snake siblings.

The development and the progressive differentiation of adaptive immunity is a characteristic of vertebrates. There seems to be almost no doubt that an immunoglobulin transmission via the egg is not restricted to *Elaphe* snakes, but is a phenomenon occurring in all egg-laying reptiles. Uller et al. (2003) expected the occurrence of a vertical immunity transfer in lizards to explain the family effects on disease susceptibility observed. Obvious elucidation for the partial hibernal antibody formation incompetence of snakes described by Zapata et al. (1992) can be given if taking notice of the purpose of an antibody transfer. The phenomenon has also significant applied aspects as it provides a simple explanation of the amazing ability of the immune system of vertebrates to adapt rapidly to new hygienic environments, e.g. in captivity.

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