Kinetics of IgY Formation after Immunisation of Hens with Different Protein Antigens

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Summary
Hens (White Leghorn) were immunised with different protein antigens, and antibodies were isolated from eggs by PEG precipitation. To determine experimental conditions under which optimal antibody titres are reached the antigen dose and the mode of application were varied. Injecting the antigen three times every four weeks in the absence of adjuvant led to a phase like immune response. A plateau of the antibody titre was only reached when immunisation was carried out in the presence of adjuvant. Antibody titres obtained from mammalian sera are equally high as those obtained from hen eggs. To label isolated IgY preparations with horse radish peroxidase no further purification or concentration steps are necessary.

Zusammenfassung: Kinetik der IgY Bildung nach Immunisierung von Hühnern mit verschiedenen Protein-Antigenen. Weiße Leghorn-Hennen wurden mit verschiedenen Proteinantigenen immunisiert. Durch PEG-Präzipitation konnten aus den Hühnereiern vitelline Antikörper isoliert werden. Um die experimentellen Bedingungen für die Ausbildung optimaler Antikörpertiter zu definieren, wurden sowohl die Antigendosis als auch der Applikationsmodus variiert. Drei Antigeninjektionen im Abstand von jeweils vier Wochen führten zu einem phasenartigen Verlauf der Immunantwort. Ein Plateau des Antikörpertiters wurde nur erhalten, wenn die Immunisierung in Anwesenheit von Adjuvans durchgeführt worden war.
Mit aviären vitellinen Antikörpern konnte ein ähnlich hoher Antikörpertiter erreicht werden, wie von Säugerseren her bekannt ist.
Um die isolierten IgY-Präparationen mit Peroxidase zu markieren, waren keine vorausgehenden Reinigungs- oder Konzentrierungsschritte notwendig.

Keywords: IgY, protein antigen, titre, kinetics, HRPO-labelling

1 Introduction

Already in 1893, Klemperer showed that egg yolk contains unusual high amounts of specific antibodies. Only in the last two decades when alternatives to conventional animal studies received attention one became aware of this source of antibodies and the possibility to use them as an alternative to antibodies produced by the conventional „bleeding" way. To obtain antibodies from egg yolk imposes, except for immunisation, no experimental stress on the animal and thus renders this method suitable to contribute to animal experiments in which manipulations are minimised. There are many reports demonstrating that large quantities of antibodies can be extracted from egg yolk (Polson et al. , 1980; Schade et al., 1992; Akita, 1993). Isolating IgY antibodies from egg yolk provides an easy and effective method by which antibodies can be obtained that are equal in quality and quantity to those prepared in the conventional way.

Here we show the kinetics of an antibody response in hens after several immunisations, carried out for various time-periods with different antigens. By the use of ELISA, the influence of the immune protocol on IgY formation is shown, and suggestions as to the appropriate immune protocols are given. Furthermore, we show that the antibodies can be used as directly labelled secondary reagent when coupled to peroxidase.

2 Materials and methods

2.1 Immunisation

Laying hens (White Leghorn), 21 weeks old, were immunised three times (day 0, 28, 56) with protein antigens intramusculary. The antigens DNP-BGG (dinitrophenylated bovine gamma globuline), mouse IgM or mouse IgG (Sigma, Deisenhofen, Germany) were used either emulsified with an equal volume of complete (PIR) or incomplete (SIR, TIR) Freund´s adjuvant (Difco, Detroit, MI) or diluted in physiological saline. The antigens were injected three times at a dose of 0.1 mg, 0.5 mg and 1.0 mg. For 100 days eggs were collected and stored at 4°C until antibodies were extracted from each egg separately by PEG precipitation according to Polson and von Wechmar (1980).

2.2 Evaluation of the titre of specific antibodies by ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed using different antigens (Nakane and Kawaoi, 1979; Kemeny, 1991, 1992). Nunc Maxisorp microtitre plates (96 well) were used as the solid support. Wells were coated with 10µg/ml PBS, and incubated overnight at room temperature. After washing three times with PBS/Tween (0.05% Tween 20) plates were incubated with the appropriate dilutions of IgY (starting at 1mg protein/ml) for 1h at room temperature. Plates were then washed three times with PBS/Tween and 50µl of rabbit anti-hen IgY coupled to horse radish peroxidase (HRPO) (Chemicon, Temecula, CA; 1:5.000 in PBS/Tween) was added to each well. After 1h the plates were washed and 50 µl freshly prepared substrate solution (ABTS, Sigma, Deisenhofen, Germany) was added. Ab-

sorbance was read at 405 nm using an ER-400 ELISA reader (SLT Labinstruments, Crailsheim, Germany). The titre was defined as the dilution at which the OD (405 nm) was threefold higher than the control values.

2.3 ELISA for determination of antigen specific binding to IgY-HRPO-conjugates

The IgY preparations were conjugated as described by Nakane and Kawaoi (1974, 1979). To determine the titre of HRPO-labelled hen anti-mouse IgG (HAM-IgG-HRPO) and HRPO-labelled hen anti-mouse IgM (HAM-IgM-HRPO) ELISA-plates were coated with protein antigens. The titre was defined as described above.

3 Results

When immunising hens every 4 weeks with low amounts of DNP-BGG, considerable variations as to the BGG-titre are observed (fig. 1a). The low immune reaction obtained in the absence of FCA can be considerably improved by using FCA/FIA.

On day 14, a specific titre of 1:3 is reached in the absence of FCA whereas in the presence of FCA, it increases to 1:200. During secondary and tertiary immune responses titres of 1:100 (0.1 mg in absence of FCA) and 1:1.000 (0.1 mg in presence of FCA) can be obtained.

Fig. 1b shows the kinetics of the immune response (measured as anti-BGG titre) after application of 0.1 mg, 0.5 mg and 1.0 mg DNP-BGG in the presence of FCA. Increasing the antigen dose by a factor of 10 results in a raise of the antibody titre from 1:1.000 to 1:6.000. During SIR and TIR plateaus can only be observed when adjuvant and doses of 0.5 mg and 1.0 mg DNP-BGG are applied.

Fig. 2 shows the antibody production against mouse IgG (A, B) and mouse IgM (C, D) after injecting 0.5 mg antigen three times. As tested by ELISA, specific IgY antibodies against mouse IgG as well as mouse IgM increase when applied with adjuvant (B, D). Maximal titres of 1:100.000 are reached. Whereas mouse IgM induces a rapid immune response on day 28 when given twice, the antibody production in response to mouse IgG is retarded. There is a remarkable increase of the immune response before day 40 (B). On day 14 titres of the primary immune response can reach values between 1:3.000 and 1:20.000.

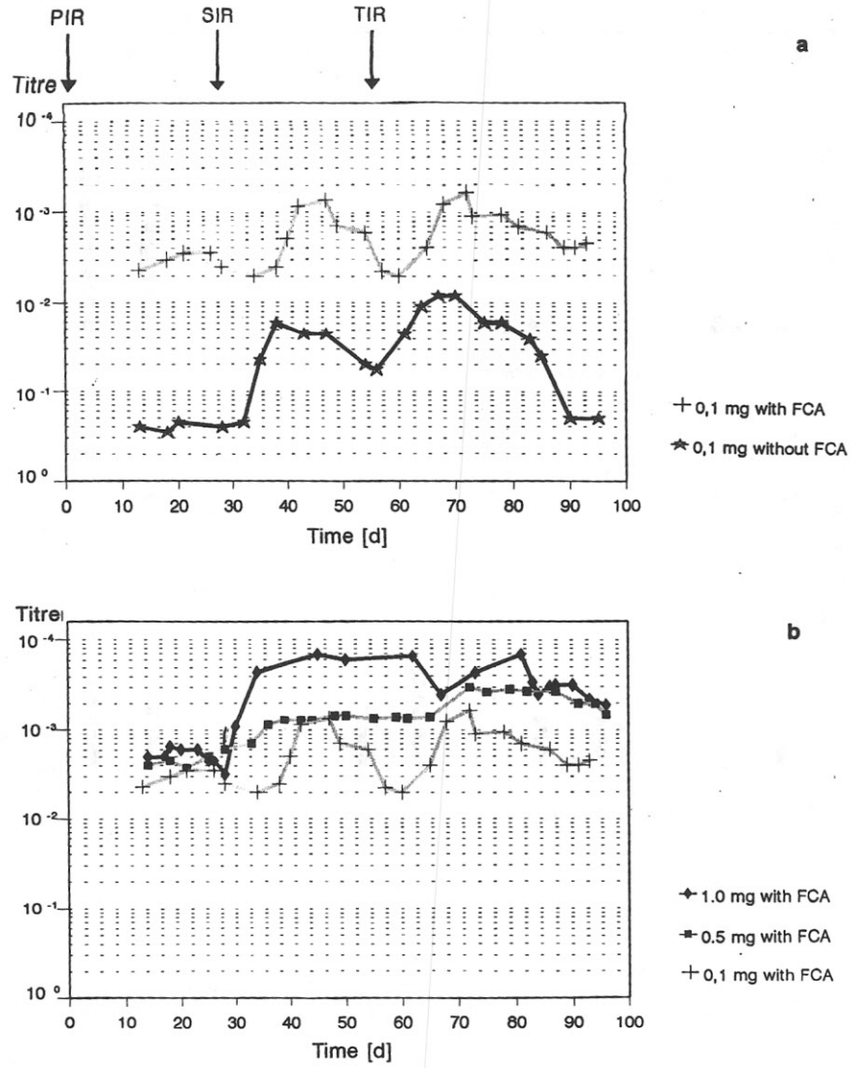

Figure 1: a: Comparison of the kinetics of the immune response following application of 0.1 mg DNP-BGG in the presence or absence of FCA. b: Comparison of the kinetics of the immune response following application of different concentrations of DNP-BGG (0.1 mg, 0.5 mg, 1.0 mg) in the presence of FCA.

Comparing the maxima of the antibody production (fig. 3) it is obvious that in the absence of adjuvant, antibody production against IgM is higher than against IgG. The hen anti-mouse IgM immune response can be slightly increased by raising the antigen dose. FCA/FIA induces an increase of both the hen anti-mouse IgG as well as the hen anti-mouse IgM response.

To use hen anti-mouse IgG and hen anti-mouse IgM as direct-labelled secondary reagents, IgY preparations obtained at different periods of the immune response were labelled with horse radish peroxidase (HRPO) according to Nakane and Kawaoi (1974, 1979). Fig. 4a and 4b show the specific binding capacity of single preparations after HRPO labelling. Titre values obtained by a direct ELISA are similar to those obtained by an indirect ELISA carried out before labelling. The data confirm previous results showing that antibodies against mouse IgG and mouse IgM obtained after immunisation with low and medium amounts of antigen in the presence of FCA are of high specific titres. Titres of hen anti-mouse IgM HRPO conjugates are high-

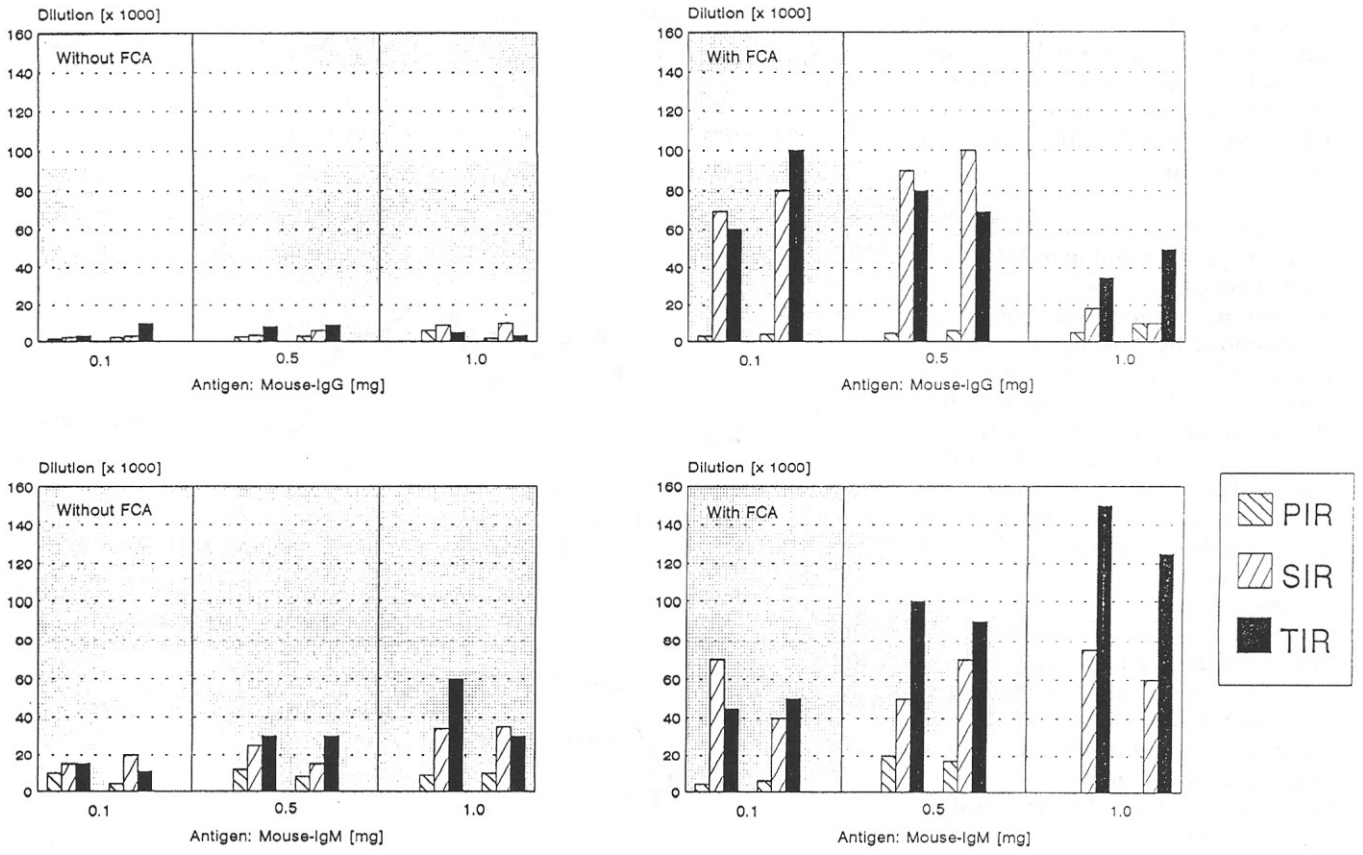

Figure 2: Maxima of titres of the primary, secondary and tertiary immune response following application of different antigen doses (0.1 mg, 0.5 mg, 1.0 mg) of mouse IgG and mouse IgM in the presence or absence of FCA. Data of two separate experiments are shown.

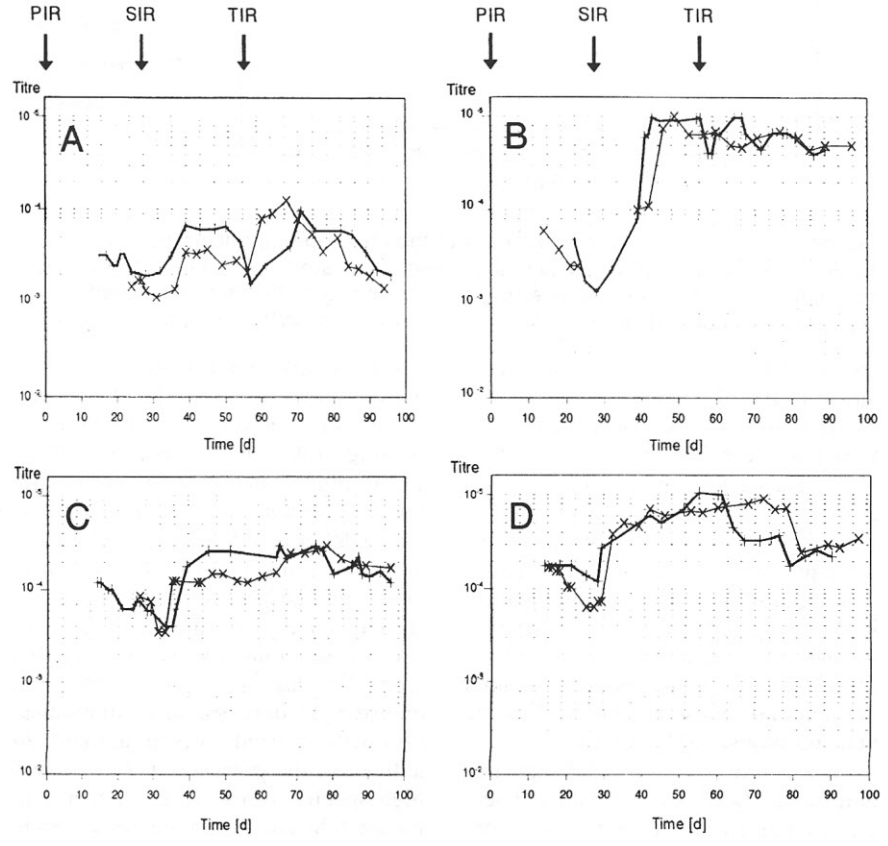

Figure 3: Kinetics of the immune response of two hens treated three times with 0.5 mg antigen (A: IgG in the absence of FCA, B: IgG in the presence of FCA, C: IgM in the absence of FCA, D: IgM in the presence of FCA)

er than those of hen anti-mouse IgG HRPO conjugates.

4 Discussion

Our experiments clearly show that White Leghorn hens are well suited to produce IgY antibodies. Young hens (SPF: specific pathogen free standard) produce more antibodies in response to the antigen than older laying hens. Preparation of IgY antibodies from egg yolk is very effective and does not

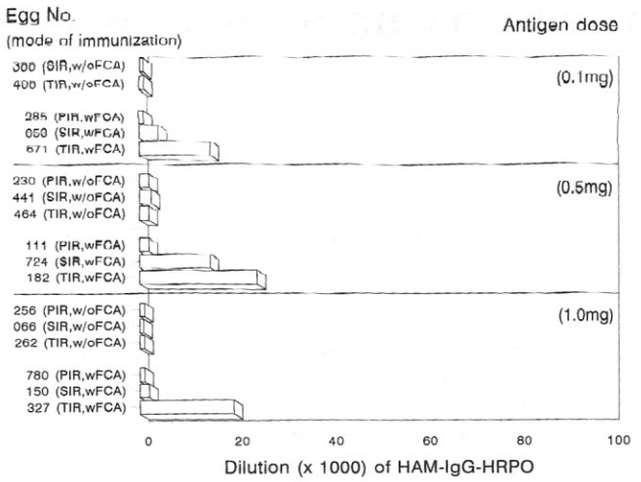

Figure 4a: Specific binding of HRPO-labelled avian vitellin antibodies directed against mouse IgG. Comparison of data obtained from single egg preparations at different phases of the immune response.

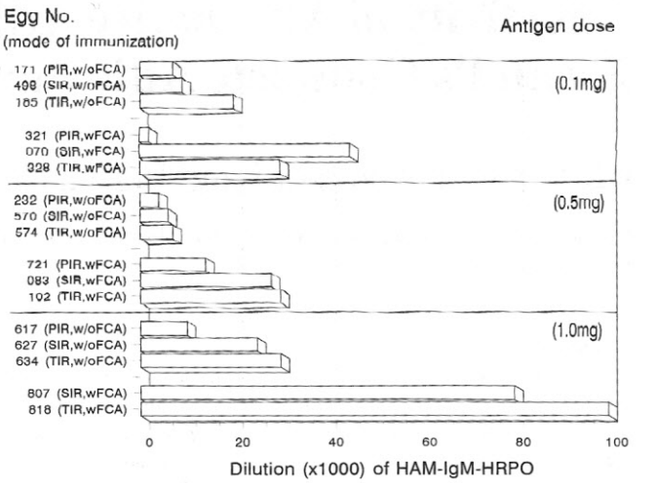

Figure 4b: Specific binding of HRPO-labelled avian vitellin antibodies directed against mouse IgM. Comparison of data obtained from single egg preparations at different phases of the immune response.

require difficult techniques. Depending on the age of the hens and the phase of the immune response, the protein content of the egg yolk varies from 2.5-7.5 mg/ml. The protein concentration does not necessarily correlate with the antibody concentration. Whereas during an experimental period of 100 days the yolk volume increases from 13 to 20 ml, the antibody content does not increase to the same extent.

To obtain specific IgY antibodies in optimal and sufficient amounts, i.e. for the use of secondary reagents, kinetics of the antibody production were carried out. While varying the antigen dose and mode of antigen application, the immunisation protocol was kept constant. When injecting the protein antigens three times every 28 days the titres of the antibodies changed periodically. This course was influenced by adding adjuvant which also enhanced the immune response immensely. Especially at times between the primary and secondary immune response an increase of the antibodies titre could be observed. A tertiary immunisation often results in maintaining the high antibody titre. Injecting three times different (low, medium or high) amounts of high molecular protein antigens showed that in the absence of adjuvant the titre did not depend on the amount of antigen administered. Mouse IgG, when given at low and medium amounts in the presence of FCA/FIA led to a raise of the titre of specific antibodies. Under the same conditions a raise of antibodies against IgM was also observed when increasing the antigen dose. Mouse IgM seems to be more immunogenic than mouse IgG. It should be avoided to inject more than 1 mg antigen since this concentration may lead to a diminished egg production. It seems that shortening the time between antigen applications has the advantage to reach higher titres and higher specific antibody concentrations after the SIR already. Thus the duration of the experiment can be shortened and the costs reduced.

The results clearly show that hen anti-mouse immunoglobulin antibodies from different egg preparations are well suited to be used as secondary reagents for a direct and indirect ELISA before and after labelling with HRPO. Coupling does not influence the specific binding capacity. Even without separating the unspecific from the specific IgY antibodies the concentration of specific IgY is sufficient to gain conjugates leading to high titres. This quick and simple method to obtain antibodies can be readily recommended.

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