Zbl. Bakt. Hyg. A 267, 247–253 (1987)

Comparative Studies on the Purity and Specificity of Yolk Immunoglobulin Y Isolated from Eggs Laid by Hens Immunized with Toxoplasma gondii Antigen

A. HASSL, H. ASPÖCK, and H. FLAMM

Abteilung für Medizinische Parasitologie (Leiter: Univ.-Prof. Dr. *H. Aspöck*)
des Hygiene-Instituts der Universität Wien (Vorstand: Univ.-Prof. Dr. *H. Flamm*)

With 3 Figures · Received February 14, 1987 · Accepted June 22, 1987

Abstract

Eggs from immunized hens were used as a source of yolk antibodies directed against soluble proteins of *Toxoplasma gondii*. The yolk immunoglobulins (IgY) were extracted and purified by the use of three different methods. The antibody yield and the purity of each batch were determined by protein measurements, gel filtration and isoelectric focusing. A sequence of two precipitation steps, i.e. a precipitation by polyethylene glycol followed by an alcohol treatment, was shown to be the most effective purification method. The specificity of the yolk antibodies was evaluated by means of an indirect hemagglutination assay, an immunodiffusion test and an immunoelectrophoresis. It was compared with the specificity of IgG antibodies obtained from sera of hyperimmunized rabbits. The precipitation patterns of IgY and IgG antibodies were non-identical. This result indicates differences between the specificities of egg yolk IgY antibodies and rabbit IgG serum antibodies, although both animal species had been immunized with identical antigen preparations.

Zusammenfassung

Legehennen wurden mit einem löslichen Extrakt aus *Toxoplasma gondii*-Trophozoiten immunisiert. Drei verschiedene Verfahren wurden angewandt, um aus den Eiern dieser Hennen die Dotter-Immunglobuline (IgY-Antikörper) zu isolieren und zu reinigen. Die Menge an Antikörpern und die Reinheit der Fraktionen wurden mittels Proteingehaltsmessungen, einer Gelfiltration und einer isoelektrischen Fokusierung bestimmt. Die höchste Antikörperreinheit erzielte man durch ein Zweistufen-Verfahren, das eine Polyethylenglycol-Präzipitation mit einer Alkoholfällung verbindet. Die qualitative Überprüfung der Antikörper erfolgte an Hand eines indirekten Hämagglutinationstests, einer Immundiffusion und einer Immunelektrophorese. Die Spezifität der IgY-Antikörper wurde mit jener von IgG-Antikörpern aus den Seren hyperimmuner Kaninchen verglichen. Dabei zeigte sich, daß IgY- und IgG-Antikörper partiell unterschiedliche Antigenkomponenten präzipitierten; daß also ein Unterschied zwischen der Spezifität von Eidotter- und der von Kaninchenantikörpern bestand, obwohl beide Versuchstierarten mit der gleichen Antigenpräparation immunisiert worden waren.

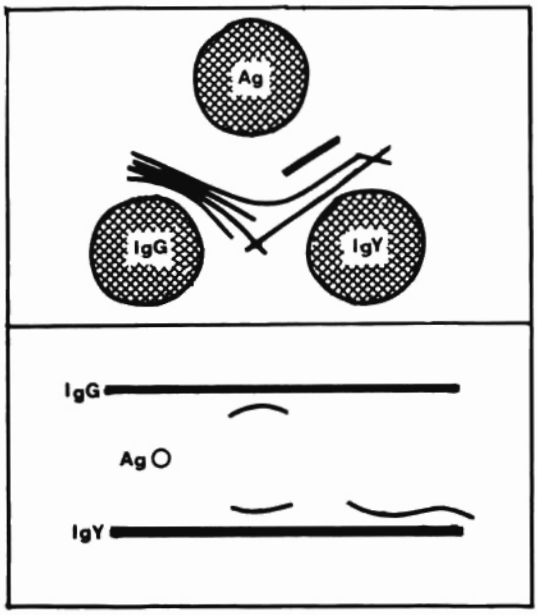

Fig. 3. Diagram of immunoprecipitation tests showing partial immunological identity between the precipitant system formed by rabbit IgG and *Toxoplasma gondii* antigen and between that formed by yolk IgY and antigen.

between pH 4.3 and pH 8.3. In Fig. 3 the precipitation patterns of the immunreactions are shown. Four precipitation bands were formed by the reaction of the antigen with the specific IgG antibodies, whereas the antigen-IgY reaction produced 3 bands. Only one antigen component was recognized by IgG antibodies as well as by IgY antibodies.

Discussion

The preparation of yolk immunoglobulins from eggs laid by previously immunized hens has been described as a convenient and inexpensive way for the production of specific antibodies (e.g. *Gottstein* and *Hemmeler*, 1985). Different methods for the extraction of the immunoglobulins from yolk have been developed, yet some of them are of limited value for routine processing as the final yields of active antibodies are low (*Jensenius* et al., 1981; *Bade* and *Stegemann*, 1984). A frequently used method for IgY separation is a precepitation process using PEG 6000 (*Polson* et al., 1980). Basing thereupon an improved procedure isolating purer immunoglobulins has recently been developed (*Polson* et al., 1985). *Bade* and *Stegemann* (1984) described an isolation method starting with a propanol precipitation step. They recommended their method as more rapid and more convenient. The aim of our study was the evaluation of the most useful one for large-scale production of specific antibodies. Particularly, we were interested in the purity, the activity, and the specificity of the yolk antibodies. A comparative study of the specificity of yolk antibodies and of IgG serum antibodies produced by rabbits should help to clarify the interchangeability of these two antibody classes.

Our results indicate striking differences in the quality of the investigated isolation procedures. The yields of specific antibodies, measured by titration in an IHA and by integration of the peak areas, are about the same in all batches indeed, but the protein contents of the batches are considerably unequal. Hence the three methods compared yield almost the same quantity of antibodies, but they do not equally purify them. The quantities of the residual yolk proteins can be estimated by the results of protein measurement and in the elution profiles of the gel filtration experiment, with certain qualifications. Whereas gel filtration may prove to be very useful for the removal of these residual proteins, a fractionation of yolk proteins according to their isoelectric points is not likely to lead to the development of a satisfying purification step as yolk protein bands are very close and linked in IEF.

Our results agree very well with data available in literature. *Bade* and *Stegemann* (1984) examined the quality of their isolation method in a PAGE. They demonstrated a considerable amount of residual protein with low molecular mass, and they recommended an additional gel filtration step for the removal of it. The high purity grade of the antibodies isolated according to *Polson* et al. (1985) is demonstrated by results of an ultracentrifugation experiment (*Polson* et al., 1985). A serious disadvantage of this purification method is the formation of IgY aggregates (*Polson* et al., 1985). Most probably these aggregates form the shoulder in the elution profile seen in fig. 1 (3 and C). Nevertheless, the most effective isolation method is the one described by *Polson* et al. (1985). A final purification step however – preferably by a gel filtration – is needed for the production of pure IgY antibodies in all cases. This gel filtration should be annexed to the isolation procedure according to *Polson* et al. (1980), since this working course is least expending.

Since there are significant differences in the production of mammalian and bird immunoglobulins (*Ambrosius* and *Haedge*, 1982), the specificity between rabbit and yolk antibodies may differ. Although our immunization antigen as well as our experimental design for animal immunization were equal, the mammalian and the bird antibodies did not precipitate the same antigenic components (fig. 3). A similar experiment using Echinococcus antigen was done by *Gottstein* and *Hemmeler* (1985). In contrast to our results they found a good correlation of the precipitation patterns between IgY and IgG antibodies. The reason for these different results is still unclear, but factors like the use of non-specific pathogen free animals, co-immunization with host proteins and different responses of hen and rabbit immune systems may considerably influence the antibody specificity. The immunization of the laying hens with selected, purified antigen components may lead to a satisfying solution of this problem.

The difference in the specificity of serum antibodies and egg yolk antibodies may shift the specificity and, thus, the reliability of serological tests. For this reason we cannot replace the rabbit IgG by yolk IgY in our ELISA for antigen detection now. An essential condition of such a replacement seems to be the immunization of hens with the "circulating antigen" which has previously to be extracted from patients sera.

Acknowledgement. The authors gratefully acknowledge the excellent technical assistance of Mrs. *Gabriela Barnert.*

References

1. *Ambrosius, H.* and *D. Haedge:* A phylogenetic view of avian immunology. Folia biologica 28 (1982) 1–21

Results

The protein contents of the IgY solutions were about 24 mg/ml (equivalent to 120 mg/egg) in batch 1, 10 mg/ml (= 50 mg/egg) in batch 2, about 9 mg/ml (= 45 mg/egg) in batch 3, and about 8 mg/ml (= 40 mg/egg) in the control batch. No significant differences of the IHA-titres between the three isolation modi could be detected, the highest positive dilution of batch 1 was 1:4000, of batch 2 1:16000, and of batch 3 1:4000; no specific IgY could be found in the control batch. Fig. 1 shows the different elution patterns of the four IgY batches obtained by gel filtration. Batch 1 contained a very high amount of proteins with a molecular weight lower than that of antibodies. In batch 2 there was a similar, but much smaller peak of impurity. Only the isolation method according to *Polson* et al. (1985) resulted in a single protein peak. Batch 3, however, as well as the control batch contained a relatively small amount of high molecular weight proteins. No significant differences between the values of the peak area integrations could be found; thus, once again proving that the amounts of IgY isolated by the three different methods are quite equal. The IEF, the results of which are shown in Fig. 2, split the antibody batches into a number of different bands located

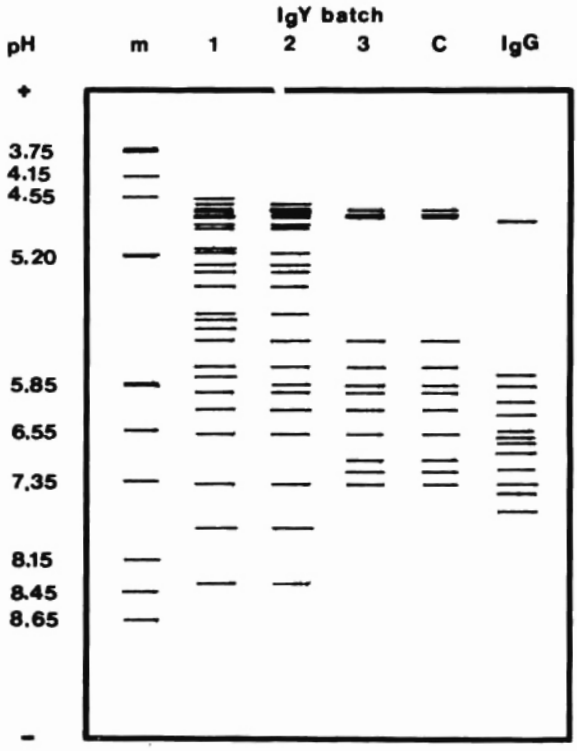

Fig. 2. Drawing of IgY-, IgG-, and marker protein bands separated by isoelectric focusing in a polyacrylamid gel of pH 3–10.
m ... standard proteins
C ... control batch

Isoelectric focusing (IEF) of IgY and IgG antibodies was performed on a Pharmacia IEF system in a pH 3–10 gradient according to the manufacturer's recommendations (Pharmacia Ges.m.b.H.). The gradient was produced in a 1 mm thick polyacrylamid gel (T5/C3) containing 6.3% Pharmalyte. Reagents for gel manufacturing were obtained from Bio-Rad Lab. (Vienna, Austria). The IgY- and IgG batches and a pI calibration kit (Pharmacia Ges.m.b.H.) were applied. Each lane was loaded with about 0.1 mg protein dissolved in a 0.1 M NaCl solution. IEF was performed at about 10 °C for 2500 Vh, afterwards the samples were stained with Coomassie Blue R–250 (Serva, Heidelberg, FR Germany).

The determination of the activity of specific yolk IgY isolated as described by *Polson* et al. (1985) and IgG antibodies from rabbit sera was done by double diffusion technique and immunoelectrophoresis. For double diffusion test a 1% agarose gel in barbital buffer (0.05 M, pH 8.6) was used; for immunoelectrophoresis we followed the technique described by *Ouchterlony* and *Nielsson* (1973). Electrophoresis was carried out by applying 100 V for 2 h. The length of the run was 5 cm. For both tests an aqueous extract from trophozoites of *Toxoplasma gondii* (strain: BK) was used as antigen. The reactants were allowed to diffuse in a moist chamber at 4 °C for 48 h. After thorough washing with PBS the precipitation bands were stained with Coomasie Blue R–250.

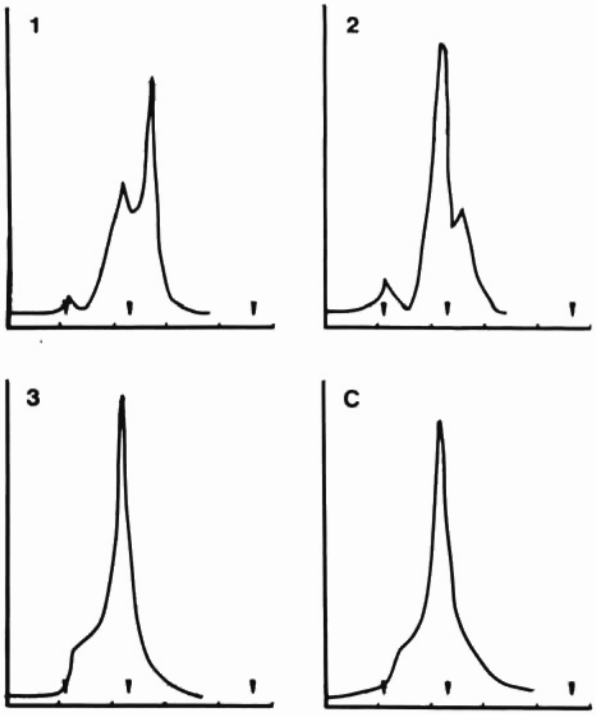

Fig. 1. Gel filtration analysis of IgY batches using Sephacryl S-300 in a 60 cm column equilibrated with 0.116 M phosphate-buffered saline. Abscissa: fractions; ordinate: relative absorbance at 280 nm. The molecular markers (arrow heads) are, from left to right, Blue Dextran (= void volume), aldolase (a.m.u. 158 kD) and L-tyrosine ethyl ester (= total volume).
1 ... specific IgY isolated by propanol precipitation (batch 1)
2 ... speficic IgY isolated by PEG precipitation (batch 2)
3 ... specific IgY isolated by PEG and ethanol precipitation (batch 3)
C... unspecific IgY isolated by PEG and ethanol precipitation (control batch)

Introduction

The use of egg yolks as an alternative source of specific antibodies and its advantages over the production and purification of specific IgG antibodies from mammals have been described by several authors (*Polson* et al., 1980; *Vieira* et al., 1984; *Gottstein* and *Hemmeler*, 1985). We have recently produced specific egg yolk antibodies (IgY) against *Toxoplasma gondii* (*Hassl* and *Aspöck*, 1986). Our aim was the purification of a substantial quantity of parasite-specific antibodies for the use in an ELISA for detection of circulating *Toxoplasma* antigens in sera (*Hassl* et al., 1987). Since several different methods for the isolation of yolk antibodies have been described (e.g. *Polson* et al., 1980; *Jensenius* et al., 1981; *Bade* and *Stegemann*, 1984; *Vieira* et al., 1984; *Polson* et al., 1985), we compared three commonly used purification methods to find out the most efficient and most convenient one. With respect to a possible replacement of conventionally raised rabbit IgG antibodies by specific IgY antibodies for certain serologic test reactions we were furthermore interested in a possible difference between the specificities of these two antibody classes. Therefore, we compared the precipitation patterns of specific IgG and IgY antibodies in two immunodiffusion tests.

Materials and Methods

Three laying hens (breed: Darco brown), 40 weeks old and conventionally kept, were immunized by intramuscular injection of 1 mg *Toxoplasma gondii* antigen together with 1 ml of complete Freund's adjuvant. The antigen was prepared by ultrasonication of *Toxoplasma gondii* trophozoites (BK strain) in aqua dest. The parasites had been cultivated and multiplied in mouse peritoneal cavities (mice: Him: OF1 ⟨Swiss⟩ SPF). They had been collected two days p.i., and washed three times in physiological saline. The hens received two booster injections at day 9 and 29 p.i. Ten eggs were collected between the 21st and the 14th day before immunization (control group); from the second week p.i. onward the eggs were regularly collected (analysis group). All eggs were stored at 4 °C until extraction of IgY. The yolks from the eggs of the control group were pooled, and a fraction of yolk antibodies was prepared according to *Polson* et al., (1985) (control batch). 15 eggs of the analysis group were broken, the yolks were pooled, and the mixture was divided into three equal parts (batch 1, 2, 3). IgY separation from batch 1 was carried out by precipitation with propanol as described by *Bade* and *Stegemann* (1984), from batch 2 by precipitation with polyethylene glycol (PEG 6000) following the method of *Polson* et al. (1980), and from batch 3 by an improved PEG-method developed by *Polson* et al. (1985). After the isolation procedure each fraction was dialysed against PBS (0.116 M phosphate buffered saline, pH 7.2 + 0.02% NaN_3). Then the protein content of each fraction was determined (Protein Assay, Bio-Rad Lab., Vienna, Austria) and the specific antibodies were titrated in an indirect haemagglutination assay (IHA) (Cellognost Toxoplasmosis; Behringwere, Marburg, FR Germany).

Specific antisera against *Toxoplasma gondii* were obtained by immunization of two conventionally raised young rabbits. They were given three intramuscular injections of 1 mg *Toxoplasma gondii* antigen together with 1 ml Freund's adjuvant on days 0, 9, and 29. The IgG-fraction was isolated from a pool serum by affinity chromatography with protein-A coupled to Sepharose according to the manufacturer's recommendations (Pharmacia Ges.m.b.H., Vienna, Austria).

A small amount (300 µl containing 3 mg protein) of each IgY batch was subjected to gel filtration through Sephacryl S–300 Superfine (Pharmacia Ges.m.b.H., Vienna). The separation was performed on a 60 × 1.6 cm column equilibrated with PBS at room temperature and a flow rate of 16 ml × cm^{-2} × h^{-1}. Extinction was measured at 280 nm. Blue Dextran 2000 [molecular weight (a.m.u.) 2000 kD], aldolase [a.m.u. 158 kD], L-tyrosine ethyl ester [a.m.u. 210 D] were used as standards. The relative content of IgY antibodies in each fraction was determined by integration of the IgY-peak areas.

2. *Bade, H.* and *H. Stegemann:* Rapid method of extraction of antibodies from hen egg yolk. J. Immunol. Meth. 72 (1984) 421–426
3. *Gottstein, B.* and *E. Hemmeler:* Egg yolk immunoglobulin Y as an alternative antibody in the serology of echinococcosis. Z. Parasitenkd. 71 (1985) 273–276
4. *Hassl, A.* and *H. Aspöck:* Untersuchungen über den Nachweis von IgY-Antikörpern gegen Toxoplasma gondii in Eiern experimentell immunisierter Hühner. Mit. Österr. Ges. Tropenmed. Parasitol. 8 (1986) 135–140
5. *Hassl, A., H. Auer, K. Hermentin, O. Picher,* and *H. Aspöck:* Experimental studies on circulating antigen of Toxoplasma gondii in intermediate hosts: criteria for detection and structural properties. Zbl. Bakt. Hyg. A 263 (1987) 625–634
6. *Jensenius, J., I. Andersen, J. Hau, M. Crone,* and *C. Koch:* Eggs: conveniently packaged antibodies. Methods for purificatin of yolk IgG. J. Immunol. Meth. 46 (1981) 63–68
7. *Ouchterlony, O.* and *L. A. Nilsson:* Immunodiffusion and immunoelectrophoresis. In: Handbook of experimental immunology, 2nd edition, D. *M. Weir* (ed.) Blackwell Scientific, Oxford (1973)
8. *Polson, A., B. von Wechmar,* and *M. H. V. van Regenmortel:* Isolation of viral IgY antibodies from yolks of immunized hens. Immunol. Commun. 9 (1980) 475–493
9. *Polson, A., T. Coetzer, J. Kruger, E. von Maltzahn,* and *K. J. van der Merwe:* Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. Immunol. Invest. 14 (1985) 323–327; New York
10. *Vieira, J. G. H., M. A. D. Oliveira, E. M. K. Russo, R. M. B. Maciel,* and *A. B. Pereira:* Egg yolk as a source of antibodies for human parathyroid hormone (hPTH) radioimmunoassay. J. Immunoassay 5 (1984) 121–129

Dr. *A. Hassl,* Univ.-Prof. Dr. *H. Aspöck,* Univ.-Prof. Dr. *H. Flamm,* Institute of Hygiene, Kinderspitalgasse 15, A-1095 Vienna, Austria.