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A simple and economical strategy for downstream processing of specific antibodies to human transferrin from egg yolk

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A rapid and economical procedure for extraction of antibodies from egg yolk is described. Laying hens were immunized with human transferrin and extracts of egg-yolk were purified with a procedure based on affinity chromatography.

The resulting purified antibodies were evaluated in a nephelometric system for the assay of transferrin in human sera. The results agreed closely with those obtained with a commercially available anti-human transferrin serum from rabbits.

Key words: Egg-yolk antibody; IgY; Immunized hen; Antibody purification; Affinity chromatography; Transferrin; Nephelometry

Introduction

The phylogenetic distance between birds and mammals, the ability of chickens to produce high levels of specific antibodies following immunization with mammalian proteins, and the transfer of large amounts of these antibodies from the serum of the laying hen to the yolk of the unfertilized egg have led to the development of several methods for extracting immunoglobulins from egg yolk (Polson et al., 1980; Jensenius et al., 1981; Bade et al., 1984; Vieira et al., 1984; Polson et al., 1985; Hassl et al., 1988).

The idea of using avian antibodies in immunoassays instead of antisera of mammalian origin is attractive for reasons associated both with the production of the antiserum and with the configuration of the assays. Indeed, antibodies can be obtained from immunized animals without the need for an invasive technique by the simple collection of eggs. It has also been demonstrated that antibodies in egg yolk are stable over a long period of time when stored at 4°C (Jensenius et al., 1981) and that large amounts of antibody, greatly exceeding the potential serum output, can be obtained by extraction of the egg yolk (Gassmann et al., 1990). On the other hand, the properties of the "7 S" immunoglobulins from avians, called IgY, have been shown to be slightly different from those of mammalian IgG (Leslie et al., 1969). Indeed, IgY does not react with mammalian complement, mammalian Fc receptors, staphylococcal protein A, or streptococcal protein G (Jensenius et al., 1981). Far more important, however, is the recent finding that chicken IgY does not react with rheumatoid factor (RF), which is a major source of interference in many immunological tests (Larsson et al., 1991).

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In the present study, human transferrin, a well-conserved protein that is widely used in clinical diagnosis (De Jong et al., 1990), was chosen as a model for the production of purified specific antibodies from egg yolk. Such antibodies were then compared with a commercially available antiserum from rabbit in a nephelometric assay.

Materials and methods

Immunization

Laying hens, 24 weeks old, were obtained from the veterinary department of the University of Antwerp, and kept isolated in wire-bottomed cages. The hens were submitted to regular light cycles, and food and water was freely available.

They were regularly injected (see below) with human transferrin (Tf) (Sigma Chemical Corp.). Antigen was first dissolved in 150 mM NaCl at a concentration of 1 $\mu g/\mu l$. This protein solution was emulsified in an equal volume of complete Freund's adjuvant (CFA) (Difco). The adjuvant-mixed antigen was homogenized with a vortex mixer before injection.

The first two doses of 500 μg of Tf each were administered by intradermal injection into the wings at a 3 week interval. 2 and 4 weeks later, a further 250 μg dose of Tf was injected into the pectoral muscle. Boosts were given monthly by intramuscular injection into the pectoral muscle (two doses of 250 μg of Tf and six doses of 150 μg of Tf). Each injection was administered into multiple sites on both sides. In this way, 2900 μg of Tf were given to each hen during the 10 month immunization period.

Extraction

2 weeks after the first injection, the egg collection began. The eggs were pooled, and the extraction procedure was run once a week. The yolk was separated from the white and carefully washed with Tris-buffered saline (TBS) (1 mM Tris, 14 mM NaCl, 0.1% NaN_3; pH 7.4 (Merck)) in order to remove as much of the albumin (white) as possible. The yolk membrane was cut and the yolk poured into a glass beaker and diluted with TBS. For one yolk (15 ml), 40 ml of TBS were added. The mixture was homogenized for ten min with a kitchen mixer (Nova). Then 40 ml of chloroform were added with continuous stirring. The mixture was then refrigerated for 24 h.

By centrifugation at 4000 rpm for 30 min at 4°C in a Sorvall RC-5 superspeed refrigerated centrifuge (HVL), three phases could be separated in the centrifuge tubes: an orange organic phase at the bottom of the tubes, a semi-solid phase in the middle, and an aqueous phase at the top, subsequently referred to as supernatant A. Each phase represented about a third of the total volume in the tubes. The organic phase was discarded; the supernatant kept; and the semi-solid phase was diluted in 40 ml of TBS to extract all residual proteins and centrifuged at 4000 rpm for 30 min at room temperature. The supernatant (supernatant B) was mixed with supernatant A, and the mixture was brought to room temperature before addition of anhydrous sodium sulphate up to a concentration of 20% (w/v). Sulphate was added in small quantities and with continuous stirring until it was completely dissolved.

After 1 h of incubation at room temperature and centrifugation at 4000 rpm for 15 min at room temperature, the precipitate was dissolved in a convenient volume of TBS. Half this volume of 36% Na_2SO_4 was then added to the clear solution and after another incubation of 1 h and centrifugation as above a final precipitate was obtained, which was dissolved in a minimal volume of TBS. The extract was dialyzed against TBS in order to remove excess sulphate. Barium acetate was used to trace the presence of sulphate in the extract.

The presence of anti-transferrin in the extracts was checked by double diffusion in gel (Feinberg, 1957); precipitation of chicken antibodies was enhanced by including 5% (w/v) of polyethylene glycol (PEG 6000) in the buffer system used.

Purification

The extracts were prepared weekly, stored at 4°C, and then pooled for purification by affinity chromatography on a column of 10 cm height and 3 cm diameter (Pharmacia) packed with divinyl-

sulfone-activated agarose gel (Mini-Leak medium, Kem-En-Tec, Copenhagen, Denmark).

The column was prepared as follows: 10 ml of gel were washed ten times with distilled water, dried, and poured into a 12 ml plastic tube. A solution of human transferrin prepared by dissolving 40 mg in 2 ml of 0.5 M $NaHCO_3$ pH 8.6 was added to the dry gel. A sufficient volume of 30% polyethylene glycol 6000 (PEG) in 0.5 M $NaHCO_3$ pH 8.6 was then added to obtain a final concentration of 5% PEG. The mixture was well homogenized; 200 μl of gel were sampled and centrifuged at 2000 rpm for 1 min and the supernatant retained. The rest of the gel was mixed overnight on an end-over-end mixer (Cinco, Vel) at room temperature. Again, 200 μl of gel were sampled and centrifuged, and the supernatant retained. The gel was then diluted with an equal volume of distilled water and mixed and centrifuged as above. Again, the supernatant was kept.

To check the efficiency of the coupling procedure, the three supernatants were tested for the presence of transferrin by double diffusion in gel: no precipitation lines should be observed with the last two supernatants.

The gel was then washed three times with one volume of 0.1 M ethanolamine chloride/NaOH, pH 9 (alternatively with 0.1 M ethanolamine/HCl, pH 9). An incubation of 30 min with continuous mixing was found to be essential during the third washing step. Finally, the gel was washed three times with one volume of 0.05 M Tris/acetate pH 7.7 + 0.45 M NaCl (neutralizing buffer) and poured into the column. The column was equilibrated with neutralizing buffer before use.

Sample (extract) was applied at a flow rate of 20 ml/h and followed by washing with neutralizing buffer to remove all unbound proteins. Elution was performed with 1 mM citrate pH 3 (elution buffer) at a flow rate of 40 ml/h with constant monitoring of the absorption of the eluate at 280 nm. Protein peak fractions were collected in 10 ml tubes containing 5 ml of neutralizing buffer to avoid denaturation of antibodies.

Each fraction collected under these circumstances was mixed on a vortex mixer, and the presence of anti-Tf was determined by double diffusion in gel. Positive fractions were pooled and 0.1% (w/v) of NaN_3 was added as bacteriostatic.

A final concentrated purified extract was obtained by ultrafiltration on a Minitan S membrane (Millipore) with a molecular weight cut-off of 100 kDa.

The purified extract was then stored at 4°C. Under these circumstances, a maximum volume of 400 ml of extract could be purified in one run.

After elution, the column was reequilibrated with neutralizing buffer. If the column was not to be used immediately, 0.1% NaN_3 was added to the neutralizing buffer as a preservative.

All the chemicals used for the extraction and purification procedures were analytical grade and obtained from Merck.

Nephelometry

The following reagents were obtained from Behringwerke: N grade reaction buffer, N grade antiserum to human transferrin and N grade protein standard serum.

0.9% NaCl and 0.05% Tween 20 in distilled water were used as diluent and rinsing solution, respectively.

Assays were performed on a Behring nephelometer 100 analyzer, connected to a Behring nephelometer 100 terminal, which permits automatic calculation of results from a stored reference curve.

Purified as well as non-purified egg yolk extracts were tested as antisera for the determination of transferrin in human sera and compared to the rabbit antiserum from Behring.

In order to obtain comparable results, reference curves for the different antisera were set up simultaneously by analysing five serial dilutions of the same batch of N protein standard serum, containing 317 mg/dl of transferrin. The calibration curves were based on a logit-log function.

A determination of Tf was performed on 268 serum samples. Samples were diluted 1/20 and 10 μl of this dilution were mixed with 80 μl of reaction buffer in a reaction cuvette. An appropriate volume of specific antiserum and reaction buffer were then added and an initial measurement of light scattering was performed 10 s after the preparation of the reaction mixture. A ‘fixed

time' method was used with a second measurement after 6 min. A comparison of the test parameters for the different types of antisera is shown in Table I.

To evaluate the stability of the antisera and the validity of the calibration curves, a commercially available control serum (Autonorm Human, Nycomed) was analysed with each series of serum samples, and at least once a day. For evaluation of the test linearity, the lyophilized Autonorm Human control serum was dissolved in various volumes of distilled water and further dilutions were made, so as to obtain a series of standards with concentrations ranging from 32 to 640 mg/dl.

Reproducibility tests were performed with the purified extract on three pools of serum samples with low (< 200 mg/dl), medium (260–340 mg/dl) and high (> 350 mg/dl) concentrations of Tf respectively.

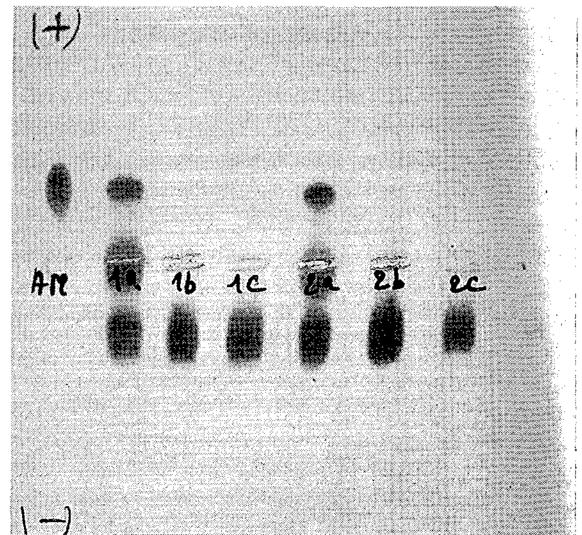

Fig. 1. Electrophoresis of egg yolk extracts prepared according to different extraction procedures. AM = albumin-Macrodex marker; a = extraction according to Kint et al. (1987); b = extraction according to the proposed procedure; c = extraction according to Jensenius et al. (1981); 1 and 2 = different weeks.

Results

In a separate run, 133 egg yolks (1990 ml), collected over the first 7 weeks, were processed according to the procedure described. The resulting crude extracts were submitted to electrophoresis and were shown to contain almost exclusively immunoglobulins (Fig. 1).

The total protein concentration in the resulting pooled crude extract (2200 ml), determined using the BCA protein assay (Pierce), was 10.9 mg/ml. The concentration of anti-transferrin antibodies in the same pooled extract, estimated by single radial immunodiffusion (RID) (Harboe et al., 1973) was 1.15 mg/ml. The total volume of concentrated extract after immuno-purification was 130 ml, with a concentration of anti-transferrin antibodies of 4.0 mg/ml.

Statistical analysis of the results of the nephelometric determination of transferrin in the series of 268 human serum samples demonstrated a linear relationship between the values obtained with the Behring antiserum and those obtained with the purified egg yolk extract ($r = 0.95$, $p < 0.01$; Fig. 2).

Moreover, a Student's t test comparison of paired samples indicated that there was no statistically significant difference between the results obtained with the two types of antibody ($t = 1.18$, $p = 0.238$). However, the differences between the results obtained with the non-purified extract and those obtained with the Behring antiserum were statistically significant ($t = 17.45$, $p < 0.01$), although a linear relationship was observed ($r =$

TABLE I

COMPARISON OF NEPHELOMETRIC ASSAY PARAMETERS

	Behring rabbit antiserum	Purified egg yolk antibodies	Non-purified egg yolk antibodies
Concentration mg/ml (RID)	1.74	4.00	1.15
Sample vol. (μl)	10	10	10
Sample dilution	1/20	1/20	1/20
Reagent vol. (μl)	40	5	80
Reaction buffer vol. 1 (μl)	80	80	80
vol. 2 (μl)	80	250	80
Measuring time	6 min	6 min	6 min

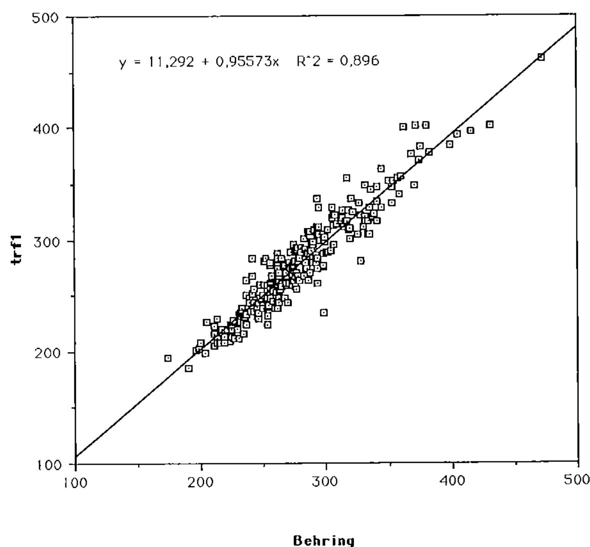

Fig. 2. Correlation between results of nephelometric determinations of transferrin using purified egg yolk antibodies or a rabbit antiserum. Results obtained on 268 human serum samples measured using either the Behring antiserum or a purified egg yolk extract (trf1).

0.97, $p < 0.01$). The mean value obtained with the non-purified extract was 292.3 mg/dl, compared to 279.6 mg/dl for the Behring antiserum.

TABLE 2

REPRODUCIBILITY STUDY OF TRANSFERRIN ASSAYS PERFORMED WITH PURIFIED EGG YOLK ANTIBODIES

Intra-assay precision, calculated from results obtained with ten replicates of the serum pools.

$N = 10$	$Pool_1$	$Pool_2$	$Pool_3$
Mean (mg/dl)	165.0	294.9	393.7
SD	8.5	10.6	8.7
CV	5.2%	3.6%	2.2%

Interassay precision, calculated from results of six runs, performed on 3 consecutive days.

$n = 6$	$Pool_1$	$Pool_2$	$Pool_3$
Mean (mg/dl)	158.7	283.9	381.6
SD	7.7	14.6	18.4
CV	4.9%	5.1%	4.8%

Using the purified extract in the analysis of the three pools of serum samples, good inter-assay as well as intra-assay reproducibility was noted. Within-run precision was checked by simultaneous analysis of ten replicates of the serum pools. To determine the interassay variation the serum pools were examined in six runs on three consecutive days. The results are summarized in Table II. Intra-assay coefficients of variation varied from 2.2 to 5.2% according to the concentration range of the serum pool. Interassay coefficients of variation were close to 5% for the three concentration levels. In the dilution experiments with the concentrated Autonorm human control serum a linear response was observed in the 80–640 mg/dl range, while paired t test results illustrated a good agreement between the observed and the theoretical concentrations ($t = 0.172$, $p = 0.87$).

Discussion

Using the immunization procedure described, significant amounts of specific antibodies were obtained as early as 2–3 weeks after the start of the immunization schedule and for up to 15 months later. However, a systematic study of antibody yield as a function of time was not performed, since crude extracts were pooled for the purification. Further experiments will be necessary in order to determine the optimal conditions of immunization.

Jensenius et al. (1981) described a relatively simple method for isolation of egg-yolk antibodies based on the extraction of yolk lipids with dextran sulphate followed by sodium sulphate precipitation of immunoglobulins. Recoveries of up to 70–80% of the total yolk IgG were obtained, while IgG constituted more than 90% of the protein in the extracts, as estimated by SDS-polyacrylamide gel electrophoresis. This clearly demonstrated that eggs from immunized chickens could provide a convenient source of antibodies for many purposes. Since then, several alternative methods have been described for the extraction of antibodies from egg yolk. Some of them are based on the separation of yolk lipids by means of lipophilic solvents (Bade et al., 1984; Kint et al., 1987) and others propose the use of polyethylene

glycol (PEG) for isolation of the IgG antibodies (Polson et al., 1985). Although each of these methods has distinct features, Lösch et al. (1986), who compared various extraction procedures, concluded that the dextran-sulphate precipitation of Jensenius was the preferred method. Indeed, certain technical aspects, such as the use of precooled organic solvents (−20°C) (Bade et al., 1984) or high centrifugal forces up to $10{,}000 \times g$ (Polson et al., 1985), may impede the applicability of some methods on a large scale.

The extraction scheme described here is derived from the method of Jensenius et al. (1981), chloroform being substituted for expensive dextran sulphate. On electrophoresis, the resulting extract was shown to be comparable to extracts obtained by the dextran-sulphate method (Fig. 1). In the extracts obtained with the procedure described by Kint et al. (1987) based on chloroform extraction alone, immunoglobulins accounted for only 50% of the total protein. This illustrates the importance of the subsequent sodium sulphate precipitation step.

In spite of the distinct theoretical advantages of using specific yolk antibodies rather than conventional mammalian antisera, only a few studies actually describe the use of IgY reagents in diagnostic tests. Most probably this is due to problems associated with antibody purity.

Consequently, although several procedures are available by which IgY antibodies can be readily extracted from egg yolk, little has been reported on the final downstream purification of these antibodies. Unlike mammalian IgG antibodies, general procedures such as affinity chromatography with protein A or protein G are not applicable to the preparatory purification of IgY. Moreover, in an optimized preparatory process, the number of steps must be minimized. Therefore, we propose a one-stage affinity chromatographic procedure with immobilized antigen (transferrin) for the final purification instead of a combination of several chromatographic processes, such as hydrophobic interaction chromatography and gel filtration (Hassl et al., 1988). The use of a divinyl-sulfone-activated agarose gel is described, but, alternatively, other types of activated resin, such as CNBr-activated Sepharose might be used successfully.

Since chicken antibodies poorly precipitate in gel diffusion analysis, PEG 6000 was incorporated in the RID gel buffer at a concentration of 5% (w/v). Alternatively, high salt concentration (NaCl 1.5 M) can be used to enhance the precipitation. The calculated recovery of specific antibodies following affinity chromatography on agarose coupled transferrin was 20.5%, as estimated by RID. The final yield of specific antibody per yolk with the combined extraction and purification step was 4.0 mg.

The yield of the combined PEG-HIC-GF procedure, reported by Hassl et al. (1988), was 14.5 mg of purified total IgY/egg. Carroll et al. (1983) obtained up to 75 mg of total IgY/egg, using a polyethylene glycol precipitation procedure. They also applied the IgY extracts to a column of antigen conjugated to CNBr-activated Sepharose B, which resulted in a recovery of 1.5 mg of specific antibody/egg yolk. The enriched antibody was shown to have a 600-fold greater specific activity than the crude IgY extract. Finally, yields of IgY up to 72 mg per egg have also been reported by Gassmann et al. (1990), using a PEG-extraction procedure followed by purification on DEAE cellulose; approximately 3.2% of the antibodies were shown to be specific.

Different methods have been described for the determination of transferrin in human serum. Among these, the direct immunological techniques have now largely replaced the indirect method of estimation based on the measurement of the maximum amount of iron the serum can bind (total iron-binding capacity)(Ramsay, 1958). Immunonephelometry (Tuengler et al., 1988) is probably one of the most interesting approaches, because of its simplicity and the possibility of automation with resulting high sample throughput and high reproducibility. The technique is based on the measurement of the increase of the forward incident light scattering intensity, caused by immune complexes that are sufficiently large.

Only minor modifications to the Behring nephelometric assay were necessary in order to permit the use of egg yolk antibodies (Table I). These modifications mainly depended on the titer of the antibodies. Indeed, the concentration of anti-transferrin antibodies in the extracts estimated by radial immunodifussion (RID) was 1.15 mg/ml

and 4.0 mg/ml respectively before and after the purification procedure, while the titer of the commercial rabbit antiserum was 1.74 mg/ml, as printed on the vial label.

The 6 min 'fixed time' method proposed in the Behring procedure was also maintained with the avian antisera: longer incubation times (multiples of 6 min) did not result in significantly increased responses, indicating that the immune reaction had almost reached its endpoint 6 min after the addition of the antisera.

Kint et al. (1987) have previously described the use of a non-purified egg yolk extract as antibody in a nephelometric assay of transferrin and reported a linear relationship between the results obtained with the chicken antibodies and a rabbit antiserum. However, the comparison was made with a very small number of samples ($n = 52$) and no statistical analyses were performed to validate the approach.

The most obvious explanation for the differences we observed, would be the presence of natural antibodies in the non purified egg yolk extract, interfering with the analysis and causing a non-specific increase of values. It should be noted that the Behring N antiserum to human transferrin is also a purified antiserum, subjected to a solid-phase immunoadsorption technique for the removal of non-specific antibodies.

Analysis of the results obtained with the Autonorm human control serum showed that deviations from the reference value were within acceptable limits (Westgard et al., 1981) during the entire observation period of 6 weeks, thereby indicating that the purified antiserum is stable and that the calibration curve is valid for at least several weeks.

This study has shown (1) that production of specific antibodies to human transferrin can easily be induced in immunized hens; (2) that large amounts of these antibodies are transferred from the serum to the egg yolk; (3) that the antibodies can be extracted from the egg yolk on a preparatory scale by means of a simple and economical procedure; (4) that the titre of the antiserum can be measured by radial immunodiffusion; (5) that the antibodies can be stored at 4° C for a long period of time without loss of activity; (6) that affinity chromatography with immobilized transferrin results in increased specificity of the antibodies. The latter is essential if the antiserum is to be used in sensitive diagnostic tests, such as nephelometric assays.

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