Extraction of a Monospecific Coombs-Reagent from Chicken Eggs

Esteban Gutiérrez Calzado[1], Eduardo Cruz Mario[1], Tomás Samón Chávez[1], Ernesto Luna Vázquez[1], Zelma Corona Ochoa[1] and Rüdiger Schade[2]

[1]LABEX, Laboratorio de Anticuerpos y Biomodelos Experimentales, CU-Santiago de Cuba

[2]Institute of Pharmacology and Toxicology, Medical Faculty (Charité) of the Humboldt-University, D-Berlin

Summary

During the last ten years the extraction of specific antibodies (ab) from the yolk of eggs of immunised chickens is more and more accepted as an useful alternative to the immunisation of mammals. The subject of this work is the immunisation of chickens with human IgG and the extraction of specific anti human IgG ab from egg yolk in order to obtain monospecific Coombs reagent. 12 Leghorn hens (25 weeks old) were immunised with intact human IgG (INTACGLOBIN®). The chickens were immunised with 100 µg IgG/animal once per week for a period of seven weeks. The highest titre was observed after the 5th immunisation, the following immunisations achieved no further titre increase. The IgY purification was performed according to the method of Akita and Nakai (1993). The resulting IgY preparation was tested for the presence of hetero-agglutinine by means of direct agglutination using human erythrocytes of all blood groups. Thereafter 58 blood donors were tested by means of direct or indirect Coombs-test using a reference reagent (DAKO) and a Coombs reagent isolated from chicken eggs (IgY antibodies). No differences have been found between the results obtained using both Coombs reagents. Presented results show that there is a possibility to produce Coombs-reagent in chickens. Advantages of this method are: 1) non invasive antibody sampling by egg collection instead of bleeding the animal (refinement of antibody production); 2) decreasing amount of animals necessary to produce high amounts of reagent; 3) IgY-preparation contains no hetero-agglutinine in contrast to serum ab from mammals, therefore additional step in reagent production e.g. the absorption of hetero-agglutinins is not necessary.

Zusammenfassung: Gewinnung eines monospezifischen Coombs-Reagens aus dem Hühnerei

Die Extraktion spezifischer Antikörper (Ak) aus dem Dotter von Eiern immunisierter Hühner als tierschonende Alternative zur Immunisierung von Säugern findet in den letzten Jahren zunehmende Akzeptanz. Ziel der vorliegenden Arbeit war die Gewinnung von monospezifischem Coombs-Reagens durch Immunisierung von Hühnern mit humanem IgG und anschließender Extraktion der IgY-Fraktion aus dem Dotter.

12 Leghorn Hennen (25 Wochen alt) wurden mit humanem IgG (100 µg Intacglobin®/Immunisierung) über eine Periode von sieben Wochen immunisiert (1x/Woche). IgY wurde aus dem Dotter entsprechend der Methode von Akita und Nakai (1993) extrahiert. Der höchste Ak-Titer ließ sich nach fünf Wochen beobachten. Testung der resultierenden IgY-Präparationen auf Heteroagglutinine mittels direkter Agglutination (alle Blutgruppen) erbrachte negative Resultate. Anschließend wurden Blutproben von 58 Spendern mittels direktem und indirektem Coombs-Test untersucht und mit einem Referenzserum (DAKO) verglichen. Es ergab sich eine 100-prozentige Übereinstimmung der Ergebnisse.

Dieses Resultat eröffnet so die Möglichkeit, Coombs-Reagens in Hühnern zu produzieren. Dies hätte mehrere Vorteile: 1. Coombs-Reagens ließe sich über eine tierschonende Technologie gewinnen. 2. Es werden weniger Tiere zur Gewinnung größerer Mengen des Reagens benötigt. 3. Das vom Huhn stammende Coombs-Reagens enthält keine Heteroagglutinine, so dass eine aufwändige Absorption des Reagens entfallen kann.

Keywords: egg yolk ab, IgY, Coombs-reagent, IgY-technology

1 Introduction

In 1945 Coombs et al. (cited after Stites et al., 1985) immunised rabbits with human whole blood and obtained a reagent able to bind to human serum-globulin. Since this time the term Coombs-serum is used for antibodies of animal origin reactive with human globulin. Later on, Coombs-serum was produced by immunising mammals using whole blood from apparently healthy blood donors or using globulin fractions obtained from whole blood. This procedure is practised up to now (Witmann, 1981; Stites et al., 1985). In addition there are reports on the use of colostrum as Coombs-reagent by immunising cows with human whole blood. Since a few years monoclonal ab with respective specificities are obtainable (e.g. DAKO, Denmark). However, up to now there are no data with respect to a standardisation or validation of the production of Coombs-serum. That concerns aspects of immunisation protocols, the species of mammals chosen, use of adjuvants etc. (Fey et al., 1973; Witmann, 1981; Stites et al., 1985; Anonymous, 1992). In the last ten years the interest of many

scientists and enterprises engaged in antibody production is attracted by the IgY-technology as a reasonable alternative to the ab-production in mammals (Schade et al., 1991; Akita and Nakai, 1993; Fichtali et al., 1993; Larsson et al., 1993). The IgY-ab have some advantages against the IgG-ab. Chickens produce an enormous amount of ab in contrast to rabbits (during a month at least 1500 mg total IgY can be sampled from one laying hen compared to appr. 200 mg total IgG from a rabbit, Schade et al., 1997). Antibody sampling from hens is non-invasive via egg collection, followed by antibody extraction from yolk. Additionally, the eggs are layed continously over a long period. Due to the phylogenetic distance between birds and mammals the immune system of chickens responds well to highly conserved antigens which do not elicit strong immune response in mammals (Larsson et al., 1993; Charter et al., 1995; Warr, 1995).

In the last years IgY-ab are used in many fields of research. For instance in diagnostic of a lot of bacterial or viral diseases in human and veterinary medicine, in detection of non-allowed drugs in food, in qualitative and quantitative measurement of a lot of bioactive proteins and peptides and, increasingly, as therapeutic/prophylactic agent in veterinary and human medicine to reduce the use of antibiotics (Janson et al., 1995; Reyes and Lopez, 1996; Dieguez, 1997). Since the Fc-part of IgY bears no binding sites for mammalian complement or natural killer cells IgY does not induce a complement mediated cell lysis or an ab-dependent cell-mediated cytotoxicity (ADCC). Due to these properties IgY-ab becomes also interesting for xenotransplantation experiments. It could be proved, that IgY-ab with α-gal specificity could protect porcine endothelial cells from human complement-mediated lysis and could inhibit ADCC *in vitro* as well (Fryer et al., 1999).

2 Animals, materials and methods

Twelve chickens (Leghorn, 25 weeks old) were obtained from a commercial breeder (Cuban Centre for the Production of Laboratory Animals, CENPALAB, Havana, Cuba). Each three chickens were kept in cages (128 cm x 65 cm x 80 cm) with water and food (special diet CM 005 Al y Co, CENPALAB, Havana, Cuba) *ad libitum* according to general ethical regulations in Cuba and to a great extent to the recommendation of the 21st ECVAM workshop on IgY-technology (Schade et al., 1997). The animals were immunised each with 100 µg intact human IgG (INTACGLOBIN®, IMEFA, Cuba) once per week for seven weeks (volume 0,5-1,0 ml). The IgG-solution (phosphate-buffered saline, PBS) were mixed with Freund's complete adjuvant (FCA, first immunisation, SIGMA, Germany) or Freund's incomplete adjuvant (FIA, booster-immunisations, SIGMA, Germany) in a ratio of 3:2 (IgG:FCA/FIA).

Blood samples (500 µl) were obtained weekly by puncture of the wing vein (blood sampling were performed in order to compare the ab titre development in the blood and in the egg yolk, respectively). The eggs were collected daily, identified and stored at 4°C for further processing.

The IgY of the yolk of app. 70 eggs (eggs collected from 12 hens, 5th week after immunisation) was extracted according to the method of Akita and Nakai as described previously (Akita and Nakai, 1993). Egg yolk was mixed with distilled water (pH 5.0 by adding HCl) in a ratio of 1:9. The solution was centrifuged (2.500 rpm, 4°C, 30 min., Medifriger, Selecta, Spain) and the supernatant was concentrated by ultrafiltration (Amicon-System, cut off of the membran 100 kD, Grace, USA) to the original volume. NaCl was added to the supernatant (0.15 M) and the pH was adjusted to 7.2. To avoid bacterial contamination sodium azide was added in a concentration of 0.1%. The IgY-preparations (later on indicated as IgY-reagent) were stored at 4°C.

The monitoring of the anti IgG ab-titre was performed weekly in yolk extract and serum by a classical immunodiffusion technique (agar gel double immunodiffusion test, AGIDT or Ouchterlony-technique, see Witmann, 1981 and Stites et al., 1985). The ab-titre was calculated as mean of twelve replicates.

Prior to the use of the anti human IgG preparation for studying blood samples of patients the IgY solution was tested on the existence of hetero-agglutinins by means of direct agglutination using erythrocytes of all blood groups. 100 µl of IgY preparation was added to 100 µl of an erythrocyte preparation (2% in 0,15 mol/l NaCl-solution) of each human blood group, mixed gently and kept for one hour at room temperature. Thereafter the mixture was centrifuged for 1 min (1000 r/min) and subsequently the presence of an agglutination was assessed. Furthermore, the reactivity of the IgY-reagent (IgY-pool as described above) obtained was tested using erythrocytes coated with commercial human anti-D IgG ab (DAKO, Denmark).

Blood samples of 58 patients were used to compare the reactivity of a reference Coombs-reagent (DAKO, Denmark) with the reactivity of the IgY based Coombs-reagent. For this study both a direct and indirect agglutination test were used (Witmann, 1981; Stites et al., 1985).

3 Results and discussion

Monitoring the ab-titre development in chicken serum and yolk-supernatant it was found an approximately parallel development (Fig. 1).

According to other authors specific IgY-ab are detected with a delay of 5-7 days (e.g. Yamamoto et al., 1975; Woolley and Landon, 1995). However, the sensitivity of Ouchterlony-test used in this study might be too low to demonstrate such a delay. On the other hand, since a pool of serum and yolk-supernatant, respectively, was used for this study, probably the individual delayed occurrence of yolk IgY could no longer be observed. Despite of the difference in the occurrence of specific IgY-ab in chicken serum and yolk the chronological dynamic of ab-titre development was found to be similar in both fluids.

As other authors (Shimizu et al., 1988; Akita and Nakai, 1993), which used a similar immunisation schedule (weekly immunisation, 100 µg antigen/immunisation/animal) we have found the highest ab-titre five weeks after the first immunisation.

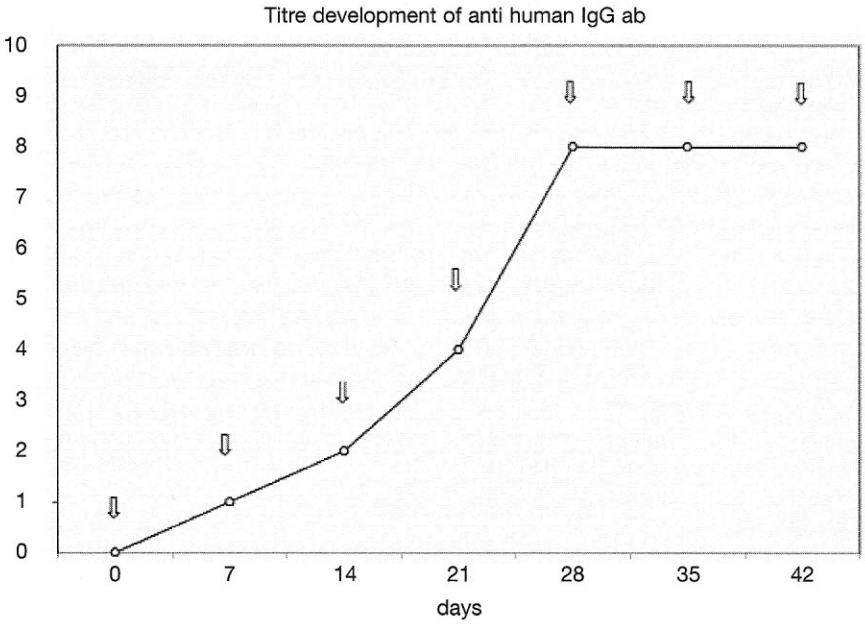

Fig. 1: Antibody titre development of hens after immunisation with human IgG. Shown is the mean value of twelve replicates. The arrows indicate date of immunisation.

According to international regulations (Anonymous 1992, Docket No.84S - 0182) the titre for Coombs reagent should be in the range of 1:16-1:64 with a free agglutination up to a dilution of 1:64 (Table 1). Following the regulation mentioned above the reagent produced in this study can be classed with group II of monospecific Coombs-reagents.

It could be proved that the IgY-ab are highly specific since the ab show no reactivity with erythrocytes of the blood groups A, B, and 0. That means, that the IgY-ab has the important advantage over mammalian antibodies used so far to produce Coombs reagent and preabsorption to remove hetero-agglutinine with reactivity against blood group antigens is not necessary.

Therefore, IgY Coombs-reagent fulfills international regulations (Anonymous, 1992) demanding no crossreactivities of immunoreagents used in human diagnostic (Table 2).

In a further investigation the blood of 58 blood donors was tested for the existence of erythrocyte bound IgG-ab. In order to investigate the reliability of the IgY-reagent a commercial Coombs-reagent (DAKO, Denmark) was used as a reference. Table 3 and 4 show, that there is no difference in the results obtained with both test systems, direct and indirect agglutination as well.

Independent on possible improvements of our biomodel (concerning in particular changes in immunisation protocol to increase the ab titre) the non-invasive IgY antibody sampling and the enormous amount of specific ab obtainable justify the introduction of this method in order to produce Coombs-reagent. In addition, if the IgY-reagent is used instead of a mammalian Coombs-reagent it is not necessary to add bovine albumin. Normally, bovine albumin is necessary as an additive to improve the immunological reaction and to avoid false results (Kerwick and Goldsmith, 1969; Sonnenwirth and Leonard, 1985). Consequently, the IgY-based Coombs-reagent is very well suited to be used as a potential immunodiagnosticum in Cuba and other countries.

Tab. 1: Agglutination of IgG-anti-D coated human erythrocytes using the IgY-reagent (IgY-pool)

Dilution of IgG anti-D ab	Dilution of IgY-reagent											
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
1/1	4	4	4	4	3	3	2	2	1	1	+/-	-
1/2	4	3	3	3	3	2	2	1	1	1	+/-	-
1/4	3	3	3	2	2	1	1	1	1	+/-	-	-
1/8	3	3	2	2	2	2	1	1	+/-	+/-	-	-
1/16	3	3	2	2	2	1	1	+/-	-	-	-	-
1/32	3	2	2	2	1	1	+/-	+/-	-	-	-	-
1/64	2	2	1	1	1	+/-	+/-	-	-	-	-	-

4 Total agglutination of the erythrocytes (one large agglutinate with bright background)
3 2 or 3 large agglutinates with bright background
2 Small agglutinates of similar size and with red background
1 Very small but distinct agglutinates with red background
+/- Very small indistinct agglutinates (unclear result)
- No agglutination
The experiment was performed in triplicate.

Tab. 2: Reactivity of the IgY-reagent in comparison with a reference Coombs-reagent (DAKO)
Indirect Coombs-test

Blood-donor	IgY anti-human IgG	Reference-anti-human IgG
1	+	+
2	+	+
3	+	+
4	+	+
5	+	+
6	+	+
7	+	+
8	+	+
9	-	-
10	-	-
11	+	+
12	-	-
13	+	+
14	+	+
15	-	-
16	+	+
17	-	-
18	+	+
19	+	+
20	+	+
21	-	-
22	+	+
23	+	+
24	+	+
25	+	+
26	-	-
27	-	-
28	-	-
29	-	-

+ or -
positive or negative agglutination reaction
The experiment was performed in triplicate.

Tab. 3: Reactivity of the IgY-reagent in comparison with a reference Coombs-reagent (DAKO)
Direct Coombs-test

Blood-donor	IgY anti-human IgG	Reference-anti-human IgG
1	+	+
2	+	+
3	+	+
4	+	+
5	+	+
6	-	-
7	-	-
8	-	-
9	-	-
10	-	-
11	+	+
12	-	-
13	+	+
14	-	-
15	-	-
16	+	+
17	-	-
18	+	+
19	+	+
20	+	+
21	-	-
22	-	-
23	-	-
24	+	+
25	+	+
26	-	-
27	-	-
28	-	-
29	-	-

+ or -
positive or negative agglutination reaction
The experiment was performed in triplicate.

4 Conclusions

The immunisation protocol used in this study resulted in specific ab suited to be used as a monospecific Coombs-reagent. Agglutination potency of the IgY-reagent and a commercial Coombs-reagent proved to be equal. Due to the phylogenetic distance between birds and mammals the IgY-reagent has the advantage not to contain natural hetero-agglutinins. Thus, a preabsorption by using human erythrocytes is not necessary. In our opinion this is an important advantage of IgY-based Coombs-reagent. In future it should be investigated if the same animal model is usable also for the production of further immunodiagnostics. There is great demand for such reagents in blood banks and immunohematologic laboratories. In addition, extensive studies are necessary to demonstrate the usefulness of IgY-based immunohematologic reagents in comparison with traditional produced reagents in order to obtain a broad acceptance. That could be an important step to refine the production of a lot of immunohematological reagents and to reduce the number of animals necessary to obtain the amount of ab needed in routine diagnostics and research.

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Correspondence to
PD Dr. Rüdiger Schade
Institut für Pharmakologie und Toxikologie
Dorotheenstr. 94
D-10117 Berlin
Tel.: +49-30-450 525 020
Fax: +49-30-450 525 919
E-Mail: ruediger.schade@charite.de