

Production of Egg Yolk Antibodies against Human Cell-Associated Antigens

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Summary

The aim of this study was the production of specific IgY against cell-associated, highly conserved mammalian proteins. CD 14 is expressed on monocyte surfaces and was identified as endotoxin receptor. P 23 is a cellular protein with unclear biological function. The induction, preparation and characterisation of egg yolk antibodies against these antigens are described.

Keywords: egg yolk antibody, IgY, cell-associated antigens

Zusammenfassung: Herstellung von Dotterantikörpern gegen humane zellassoziierte Antigene.

Das Ziel unserer Untersuchungen war die Herstellung von spezifischen IgY gegen zwei zellassoziierte, hoch konservierte Proteine der Säuger. CD 14 wird an der Zelloberfläche von Monozyten exprimiert und stellt einen Rezeptor für Endotoxine dar. P 23 ist ein zelluläres Protein, dessen biologische Funktion weitestgehend unaufgeklärt ist. Die Induktion, Präparation und Charakterisierung von Dotterantikörpern gegen diese beiden Antigene wird beschrieben.

1 Introduction

Polyclonal antibodies are typically isolated from serum of mammals after an immunisation procedure. Egg yolk antibodies are a real alternative to mammalian antibodies in several diagnostic tests and analytical systems based on immunological reactions. Many studies have demonstrated the production and application of avian antibodies against various antigens, especially virus and bacterial antigens, serum proteins and peptides (Gassmann et al., 1990; Schade et al., 1991).

The present study describes the induction of specific antibodies against two cell associated antigens (CD 14, p 23) in laying hens, the separation of the IgY and first characterisation experiments.

The 53 kDa glycoprotein CD 14 is expressed on monocyte surfaces and was identified as an endotoxin receptor (Wright et al., 1990; Schütt et al., 1992). Endotoxins (lipopolysaccharides, LPS) are constituents of the outer membrane of gram-negative bacteria. They induce a great variety of patho-immunological effects in higher organisms. Endotoxins play a central role in the immune reactions of trauma, shock, and sepsis (Freudenberg et al., 1993).

The binding of endotoxin (LPS) to CD 14 was mediated by so-called LPS-binding protein (LBS) present in serum. After the activation by endotoxin, monocytes produce free oxygen radicals, IL-1, IL-6, TNF alpha and other mediators (Schütt and Schumann, 1993).

CD 14 is also present in serum in a soluble form (sCD 14). Both forms differ slightly in molecular weight (48 kDa and 53 kDa). The immunophysiological or pathophysiological function of the serum CD 14 (sCD 14) remains unclear (Grunwald et al., 1993).

The p 23 is a cellular protein and it is described as „mammalian growth-related protein“. A high induction of the synthesis of this protein was observed after serum stimulation of mouse fibroblasts and Ehrlich ascites tumour cells (Bommer et al., 1994). The protein is probably phylogenetically conserved in mammals, but the biological function of p 23 is not clear.

2 Animals, materials and methods

2.1 Antigens

We used a recombinant human soluble CD 14 as antigen for the immunisation procedure. Briefly, CHO-cells were transfected with human CD 14 cDNA

cloned into the expression vector pPOL-DHFR and selected and cultured as previously described (Jack et al., 1995). sCD 14 was isolated from the culture medium of CD 14 transfected CHO-cells by affinity chromatography. The anti-CD 14 monoclonal antibody biG-2 (biometec GmbH Greifswald, Germany) was coupled to Eurocell ONB carbonate (Knauer Berlin, Germany) according to the manufacturer's instructions.

The p 23-antigen was a generous gift of Dr. U. A. Bommer (St. George's Hospital Medical School; Department of Cellular and Molecular Sciences; Division of Biochemistry, London).

2.2 Chickens and immunisation procedure

Laying hens were obtained from a commercial breeder at 22 weeks of age, shortly after laying had started. The hens were submitted to regular light cycles, and food and water were freely available. They were examined before immunisation for endoparasites, bacterial causal agents and for the antibody status to a panel of avian viruses.

A pre-immunisation serum sample and egg yolk sample were collected from each hen as basic and control probes.

The cell-associated antigens were mixed and homogenised (four parts antigen/one part adjuvant) with Freund's complete adjuvant for the initial immunisation or with Freund's incomplete adjuvant for the booster injection. Three boosts were given after the initial injection (fig.1,2). Hens were immunised by injection of 0,5 ml of the antigen-adjuvant mixture into the pectoral muscle on both sides.

Eggs were harvested daily and stored at 4 °C until separation of the IgY from the yolk.

2.3 Antibody purification

The egg yolks which contain the specific antibodies were separated from egg white and the yolks were washed in a stream of water. The yolk material was homogenised and was dispersed in a fivefold volume thereof with deionised water (pH 7,2). The suspension was stored at -20 °C three times. Following this, the egg yolk suspension was centrifugated (1500 xg, 20 min, 4 °C) and the clear supernatant containing the IgY was stored at -20 °C.

To isolate the IgY from the egg yolk fluid we used two methods, alone or in combinations. The first method was a two-step precipitation procedure using saturated ammonium sulfate solution (Wallmann et al., 1990; Fischer et al., 1996). The second method was the so-called „BATCH“- purification using the Bakerbond Abx-matrix (J. T. Baker B. V., Germany) described by Schade et al. (1994). The preparation effect was

evaluated by SDS-polyacrylamide gel electrophoresis of the purified IGY (here not shown). After the detection of the protein concentration (Peterson, 1979) the purified IgY was stored at -70°C until use.

2.4 Detection of the egg antibodies against p 23 by an enzyme immunoassay

Polystyrene microtiterplates (Nunc, Immunoplate F 96; Maxisorp) were used as solid phase. The wells were coated with 5 µg/ml antigen in carbonate/bicarbonate buffer, pH 9,6, (SIGMA Chemicals), and incubated 2 h at room temperature. Following this, 100 µl PBS, pH 7,4, with 1 % casein (SIGMA Chemicals) were added per well. After incubation of 1 h at room temperature the plates were washed two times with PBS containing 0,05 % Tween 20 (SIGMA Chemicals). The egg yolk antibodies were diluted (1:100) in PBS / Tween 20 / 0,1 % casein, and 100 µl per well were incubated for 1 h at room temperature. After four washings, 100 µl/well of a Peroxidase (POD)-conjugated rabbit anti-chicken IgG antibody (SIGMA Immunochemicals), diluted 1:4000 in PBS/Tween 20/ 0,1 % casein, were added in each well. Plates were incubated for 1 h at room temperature. The plates were washed four times and 100 µl/well peroxidase substrate (0,1 mg/ml 3,3', 5,5'-tetramethylbenzidine in phosphate-citrate-buffer, pH 5,0, plus sodium perborate) was added. After 15 min incubation, the substrate reaction was stopped with 50

µl/well 1M sulfuric acid and the absorbance was measured at 450 nm using an ELISA microplate reader (MR 5000, Dynatech).

2.5 Detection of egg antibodies against CD 14 by an enzyme immunoassay

Microtiterplates (Nunc, Immunoplate F 96, Polysorp) were coated with 50 µl per well of sCD 14 antigen at a concentration of 5 µg/ml in carbonate/bicarbonate buffer (pH 9,6) over night at 4 °C. The plates were washed three times with so-called washing buffer (8,0 g NaCl; 0,2 g KH₂PO₄; 2,9 g Na₂HPO₄·x12 H₂O; 0,2 g KCl; 0,5 ml Tween 20; Aqua dest. ad 1000,0 ml / pH 7,4). All further washings were carried out with this buffer. The antigen coated plates were blocked with washing buffer containing 10 % Gelafusal (Serumwerk Bernburg, Germany) and incubated for 30 min at 37°C. After washing steps, 50 µl of the egg yolk antibodies were added in each well. The avian antibodies were diluted in washing buffer plus 10 % Gelafusal. Following 1 h of incubation at 37 °C, the plates were washed three times and 50 µl/well POD-labeled anti-chicken IgG (SIGMA Immunochemicals) were added (1:20.000 in washing buffer/10 % Gelafusal). Plates were incubated for 1 h at 37°C. After this time the plates were washed and 50 µl/well of substrate chromogen solution (5 mmol/l o-phenyldiamin; 5 mmol/l H₂O₂ in citrate buffer, pH 5,0) was added. The reaction was stopped with 1M H₂SO₄

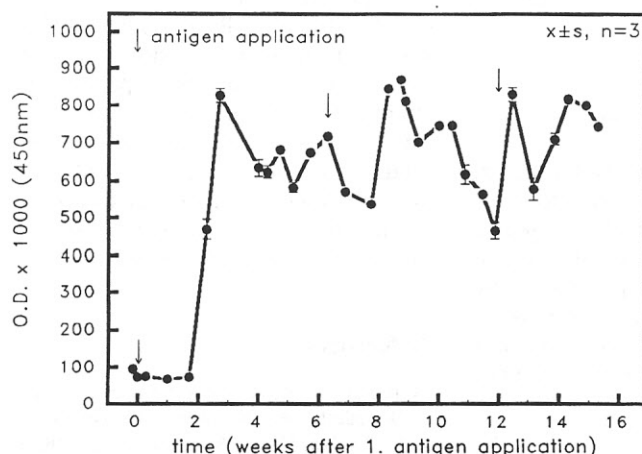


Figure 1: Time-curve of antibody titre development after immunisation with p 23.

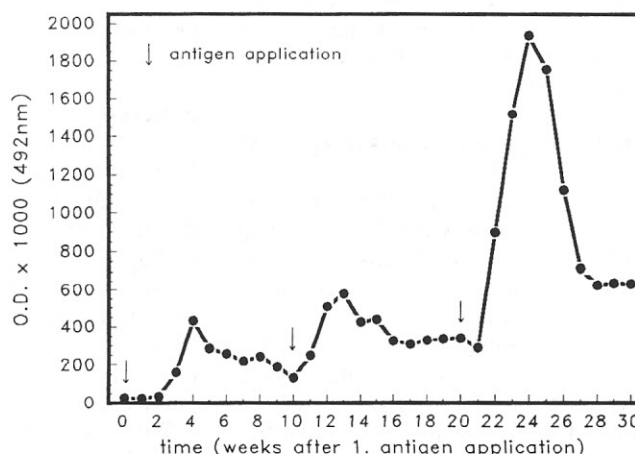


Figure 2: Time-curve of antibody titre development after immunisation with CD14.

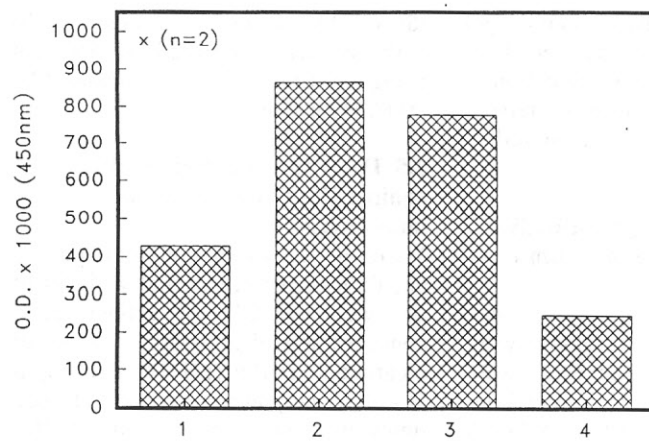


Figure 3: Comparison of different antibody preparation methods. The antigen-antibody specific binding was detected in enzyme immunoassay, based on the same protein concentration of 5 µg/ml. The protein concentrations were measured by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Germany) 1 = combination of „BATCH“-purification and precipitation procedure using ammonium sulfate; 2 = precipitation procedure using ammonium sulfate solution; 3 = combination precipitation using ammonium sulfate and „BATCH“-purification; 4 = „BATCH“-purification

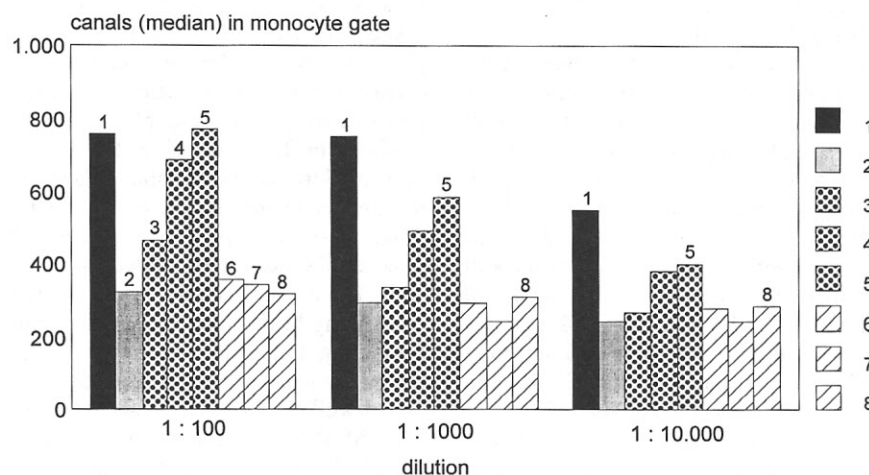


Figure 4: Comparison of binding of egg yolk antibodies and rabbit antibodies on CHO-cells, transfected with human CD 14.

1 = rabbit anti-CD 14; 2 = rabbit control antibody; 3 = avian antibody (BATCH); 4 = avian antibody (only in water solution); 5 = avian antibody (precipitation with ammonium sulfate); 6 = PBS/anti-chicken IgG-FITC control; 7/8 = unspecific avian antibodies

(50 µl/well) after 20 min. The absorbance was measured at 492 nm using a SLT-reader system (SLT Labinstrumments, Germany).

2.6 Binding experiments of egg yolk antibodies to CD 14

CHO-cells transfected with human CD 14 were adjusted with PBS containing 0.05 % NaN₃ to 5x10⁶ cells/ml. 2x 10⁶ cells were incubated with 20 µl of antibody dilution in a final volume of 70 µl for 30 min at 4°C. After washings 20 µl of FITC-labeled anti-Chicken IgG-antibody (SIGMA) was added, incubated for 30 min at 4 °C and washed twice again. The fluorescence intensity of the cells was measured by flow cytometry using a FACScan cell ana-

lyser (Becton-Dickenson Heidelberg, Germany).

3 Results and Discussion

From the experience gained with egg yolk antibodies in our laboratory, we described the production of specific IgY against two cell associated, highly conserved mammalian proteins in the present report. As far as we know, these are the first anti-sCD 14 and anti-p 23 antibodies induced in non-mammalian species.

We have immunised laying hens with the protein antigens over an extended time period. All hens showed an immune response independent of the

kind of antigen applied. The time-curve of antibody titre development shows a continuous increase of the specific IgY titre (fig. 1,2). The discrepancy of the titre development may be due to the use of different time regimens of immunisation.

Large amounts of antibodies against sCD-14 and p 23 were obtained from egg yolk by standard protein purification methods. The demonstrated methods can be performed anytime on a large scale with simple equipment.

The comparison of the purification methods demonstrate the advantage of precipitation procedure using saturated ammoniumsulfate solution (fig. 3). But these investigations were based on the whole protein contents of the preparations, not on the real IgY amounts. Previous research in our laboratory showed that the combination of these methods to be the preferred preparation procedure (Fischer et al., 1996).

Fig. 4 demonstrates the possibility of substituting mammalian antibodies against sCD 14 by the IgY. A central problem in this test system is the stability and purity of the egg yolk antibody preparation.

In present experiments we use the anti-CD 14 egg yolk antibody in different applications in comparison to antibodies from mammals, especially in immunohistochemistry. The anti-p 23 antibodies are used in characterisation studies. In special experiments we want to demonstrate the determination of the specificity of the avian antibody against different p 23 preparations.

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