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Development of an animal–human antibody complex for use as a control in ELISA

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Abstract

In order to provide the equivalent of a human anti-human protein antibody as positive control in ELISAs, a goat–human antibody complex was created using chemical cross-linking. The resulting hybrid complex had a larger molecular size on HPLC and SDS-PAGE. In ELISA, the goat–human complex bound to human antigen and was detectable by a secondary anti-human conjugate. The method to make the hybrid complex is simple, cost-effective and can be used to make human-like antibodies to many human proteins.

Keywords: Animal; Human; ELISA; Antibody; Immunogenicity; HAHA

1. Introduction

A variety of recombinant human proteins have been marketed as therapeutic drugs and administrated in patients for treatment of various diseases. Some of these protein drugs, such as recombinant Interleukin-2 (rIL-2) [1] and granulocyte colony stimulation factor (GCSF) are administered to patients for a short period of time [2]. Others such as human insulin [3] and erythropotin (Epo) [4] are repeatedly administered to patients and are intended for long-term use. When recombinant human proteins are given to patients, a concern is that some will develop antibodies to the proteins [5–7]. These antibodies may lead to the necessity for dosing at higher drug levels to offset the drug resistance [8]. It has also been shown that in some cases, antibodies to the recombinant protein cross-react with the natural human protein, leading to a deficiency disease of the hormone [9].

It is, therefore, of interest to develop assays and monitor patients' immunoresponse to the human protein drugs. Among the assays, ELISA is widely used because of its efficiency, sensitivity, accuracy and reproducibility. In these ELISA assays, a microplate coated with a therapeutic protein is incubated with patients' serum samples. A portion of antibodies specific for the therapeutic protein becomes bound to the wells. These antibodies can be detected by an anti-human secondary antibody conjugated to a reporter enzyme such as horse-

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radish peroxidase (HRP) followed by chromophoric substrates of HRP. The color produced in each well is proportional to the amount of human antibody bound to the therapeutic protein.

A positive control is necessary in an ELISA assay to insure consistent performance of the assay. This control is one or more dilutions of an antibody that binds specifically to the coated protein. Ideally this control is a human antibody, and therefore, can be detected in the same manner as antibodies from patients. However, in many cases, a human antibody or serum from a seropositive human donor or monkey is not commercially available. It then becomes necessary to use an antibody from other species, such as mouse, rabbit, or goat. The detection of this species antibody in the ELISA requires the use of a secondary antibody that is different from the one used for human antibody.

There are a few alternatives to produce human anti-human antibodies, such as phage display technology [10] and xenomouse technology [11]. However, these technologies require licensing, reengineering of phage expression constructs or manipulation of transgenic mice, which are time and labor consuming. To get around these problems, we have developed a feasible method to generate hybrid antibodies. These hybrids are antibodies raised in animals against human proteins to which human immunoglobulin has been chemically linked. This allows the production of positive control antibodies that are treated and detected as if they were human antibody. We report here the generation of a hybrid antibody to human serum albumin (HSA) and its properties in ELISA. We discuss the application of this method to the generations of similar hybrid antibodies.

2. 2. Materials and methods

2.1. Crosslinker

[*N*-Succinimidyl 3-(2-pyridyldithio)propionate] (SPDP) was purchased from PIERCE (Rockford, IL) and dissolved in dimethyl sulfoxide (DMSO), Sigma at 2 mM.

Dithiothreitol (DTT) from Sigma (St. Louis, MO) was prepared in acetate buffer (100 mM sodium acetate, 100 mM NaCl, pH 4.5) buffer at 24 mg/ml.

2.2. Immunoglobulins

Goat anti-HSA pAb was purchased from Nordic Immunology and human gamma globulin were purchased from Jackson Immunoresearch Laboratories. Human gamma globulin was used because it is inexpensive, readily obtainable and produced the greatest signals with our secondary antibody (HRP-label goat anti-human IgG, M, A).

2.3. Cross-linking the two immunoglobulins

Goat anti-HSA pAb was dissolved in phosphate buffered saline (PBS) to produce a 1 mg/ml solution. To 0.5 ml of this goat anti-HSA pAb solution, 13 μl of 2 mM SPDP in DMSO was added. The mixture was then rotated for 2 h at room temperature. To remove excess SPDP, the mixture was transferred to a mini slide-A-lyzer (PIERCE) and dialyzed in 1 liter of 1 × PBS for 24 h with three changes of the buffer.

Human gamma globulin (0.5 mg, Sigma) is also diluted to 1 mg/ml in PBS and treated with 13 μl of 2 mM SPDP in DMSO. After slow rotation for 2 h at room temperature, the mixture was transferred to a mini slide-A-Lyzer and dialyzed in 1 liter of the acetate buffer overnight with three changes of the buffer. The low pH acetate buffer was used to prevent reduction of intra-molecular disulfide bonds in the subsequent step [12,13]. 125 μl of freshly prepared DTT (12 mg DTT in 500 μl of the acetate buffer) was added to the human gamma globulin and incubated at room temperature for 30 min, then the protein was diafiltered on a centricon filter (Amicon) first with the ten volumes of acetate buffer to remove DTT followed by five volumes of PBS to replace the acetate buffer.

The SPDP modified goat anti-HSA pAb (0.5 mg) was then mixed with 0.5 mg of SPDP and the DTT modified human gamma globulin in a tube and rotated at 4 °C overnight to form the SPDP-linked hybrid complex that is on average com-

posed of one molecule of goat anti-HSA pAb and one molecule human gamma globulin.

2.4. Chromatography

HPLC (System Gold, Beckman) was used for antibody characterization and purification. Human gamma globulin, goat anti-HSA pAb and the hybrid complex were analyzed at room temperature on a G3000SW column (TOSOH Biosep) as stationary phase. Mobile phase was 1 × PBS at a flow rate of 1 ml/min, and injection volume was 20 μl for each sample. A diode array UV detector (Beckman) was used to record peaks at 215 nM. Preparative separation of the hybrid complex was carried out in the same conditions except 200 μl of the sample was injected onto the column each time. The major peak containing the antibody hybrid complex eluted around 37 min. The fractions containing this peak were collected and concentrated by centricon filters. Protein concentration of the hybrid complex was determined by the BCA method using horse gamma globulin as standard (PIERCE).

2.5. SDS-PAGE

The hybrid complex was mixed with or without DTT Sample Reducing Agent (Invitrogen) in Tris–glycine SDS sample buffer (Invitrogen). The human gamma globulin and the goat anti-HSA pAb were mixed with Tris–glycine SDS sample buffer. All samples were denatured at 90 °C for 3 min. The samples were loaded to a precast Nupage 3–8% Tris–acetate gel (Invitrogen) and ran at 60 V for 3 h. The gel was stained with Simple Blue Safe Stain reagent (Invitrogen) and destained following the manufacturer's instruction. The gel image was captured by VersaDoc Imaging System (Bio-Rad).

2.6. ELISA

PolySorp® 96 well plates (Nalge NUNC international) were coated overnight at 4 °C with HSA (Sigma) at 5 μg/ml in PBS. The plates were blocked with a sample buffer (PBS containing 1% BSA and 0.1% Tween-20) for 2 h at 37 °C.

Binding of human gamma globulin, goat anti-HSA pAb, or the hybrid complex to HSA-coated plates was measured as follows. Two-fold serial dilutions of each antibody solution were incubated at room temperature in the HSA plates (100 μl per well) in triplicate for 2 h. Starting concentrations were 4 μg/ml for human gamma globulin and the hybrid complex. Starting concentration was either 4 or 0.5 μg/ml for the goat anti HSA pAb. After washing four times with 0.1% Tween 20 in PBS, detector of horseradish peroxidase-labeled goat anti-human IgG,M,A (50 ng/ml, Jackson Immunoresearch Laboratories) (Fig. 3A) or horseradish peroxidase-labeled rabbit anti-goat IgG (50 ng/ml, Jackson Immunoresearch Laboratories) diluted in sample buffer was added (Fig. 3B). After an additional incubation at room temperature for 1 h, the plates were washed four times, and 100 μl of TMB (3,3′,5,5′ tetramethyl benzidine, KPM) and hydrogen peroxide substrate was added to each well. The color formation was stopped by addition of 100 μl of 500 mM sulfuric acid. Absorbance was measured at 450 nm in a microtiter plate reader (Molecular Devices).

3. Results and discussion

3.1. Formation of the hybrid complex

The goat anti-HSA pAb and the human gamma globulin have molecular weights in the range of 130–150 kDa on the SDS gel (Fig. 2, lane 2 and 3). On HPLC both proteins had a major peak at around 43 min (Fig. 1A, B). The goat anti-HSA pAb-human gamma globulin hybrid complex should have molecular weight of 260–300 kDa. As expected, this hybrid complex eluted faster than either component (Fig. 1C). The major peak was detected at around 38 min with a shoulder at 43 min. The ratio of peak area to shoulder allows us to estimate that approximately 30% did not form into complex. The uncomplexed antibody was largely removed by the HPLC purification (data not shown). The presence of trimers or larger complexes was not measurable under the stated conditions.

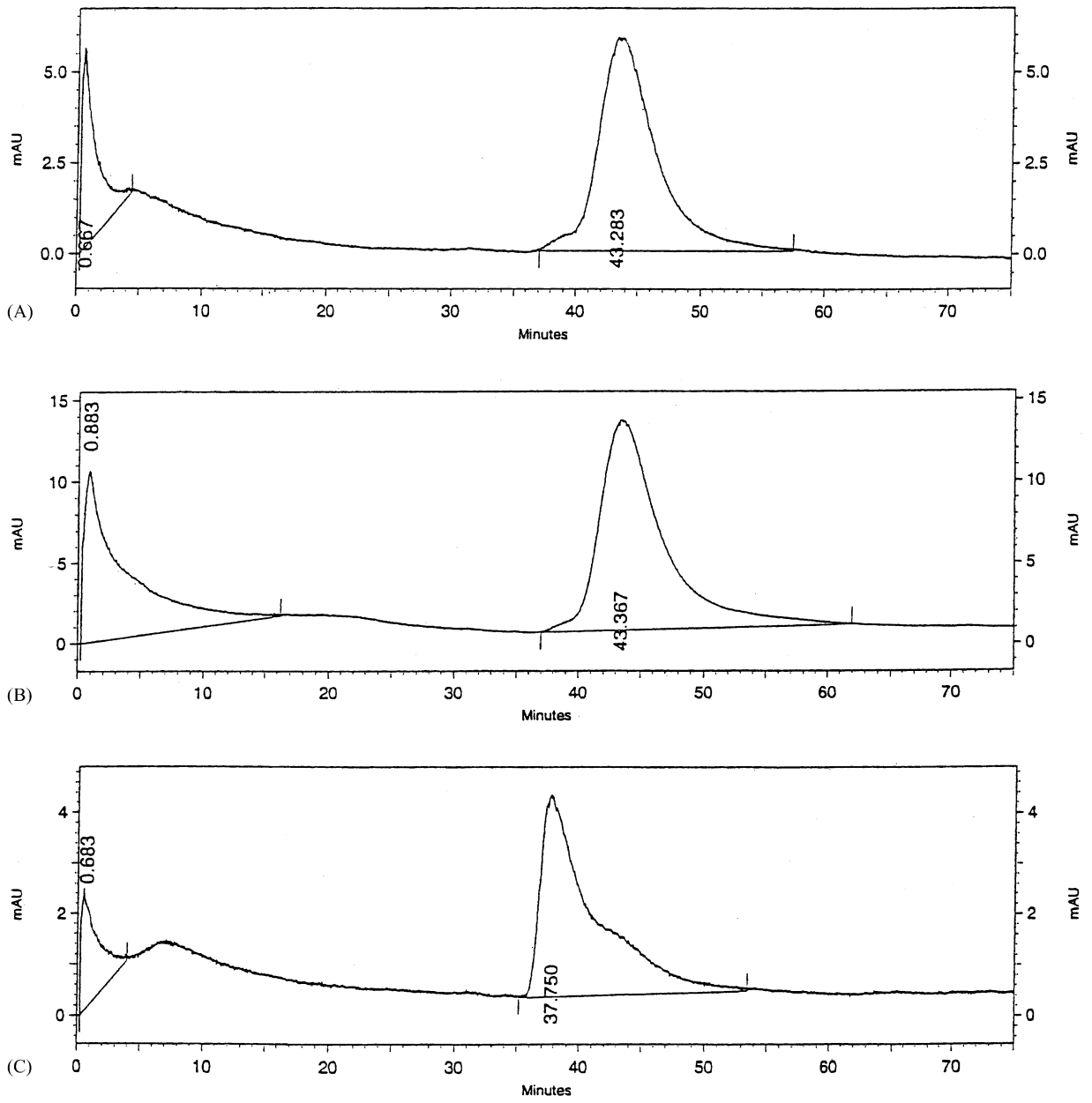

Fig. 1. Chromatograms of goat anti-HSA pAb (A); human gamma globulin (B) and the hybrid complex (C) in PBS.

3.2. Characterization of the hybrid complex

To rule out that the early peak observed in the HPLC was merely aggregations of the human gamma globulin and the goat anti-HSA pAb, the peak was analyzed on an SDS gel. Under denaturing but non-reducing conditions, the hybrid complex contained protein bands that had a minimum

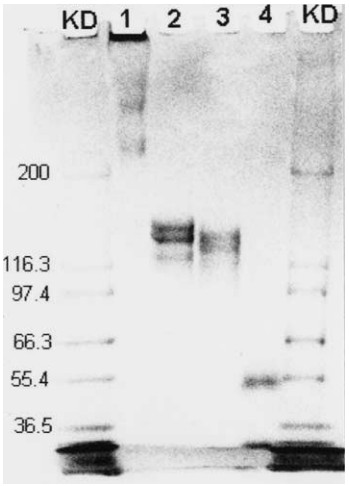

Fig. 2. SDS-PAGE gel electrophoresis of the hybrid complex and its components. Six micrograms of the hybrid complex (1), three microgram of the human gamma globulin (2) and three microgram of the goat anti-HSA pAb (3) under non-reducing condition. Six micrograms of the hybrid complex (4) under reducing condition.

estimated molecular weight of 250 kDa or larger (Fig. 2, lane 1). No visible monomers were seen in this lane, suggesting that the complex is stable and that the HPLC was effective in moving monomer. There was protein staining on the top of gel (lane 1), which is likely due to the multimers of the hybrid complex too large to enter the gel. In cross-linking, the challenge ratio of SPDP to each component was calculated to be 8:1. It is likely that at this ratio, each component was able to cross-link to more than one other antibody molecule, resulting in the formation of the multimers. Under reducing conditions, all of the higher molecular weight bands disappeared and two bands with molecular weight of 50 and 25 kDa, corresponding to the sizes of antibody's heavy chain and light chain, were produced (lane 4). This demonstrates that the human gamma globulin and the goat anti-HSA pAb were indeed cross-linked by the disulfide bond to form mixed populations of multimers. In the starting materials no higher molecular weight bands were present (lane 2 and 3).

3.3. Detection of HSA by the hybrid complex

Using a HRP-labeled goat anti-human IgG, M, A detector, no signal above background was produced in wells containing as much as 4 μg/ml of either goat anti-HSA pAb or human immunoglobulin (Fig. 3A). However, the hybrid complex was detectable down to a concentration of 30 ng/ml, as defined by $2\times$ background OD. The presence of signal from only the hybrid complex solution is further evidence that a stable complex was formed containing both goat anti-HSA pAb and human gamma globulin components.

When a HRP-labeled rabbit anti-goat IgG detector was used, both the goat anti-HSA pAb and the hybrid complex were detected (Fig. 3B). The concentration of the hybrid complex that produced a significant signal above the background was about 40 ng/ml, while the concentration of goat anti-HSA pAb was around 8 ng/ml. The loss of sensitivity in the hybrid complex solution is partially due to the difference in molecular weight. When calculated by the molar ratio, the actual loss of sensitivity in the hybrid complex solutions was much smaller. Additional loss of activity is likely caused by steric hindrance or chemical inactivation of antibody binding sites. Since the hybrid complex is intended for use in the detection by a secondary anti-human Ab detector, some loss of the detection by an anti-goat Ab detector here would not affect its utility.

There are several potential ways to increase the detection activity of a hybrid complex by an anti-human Ab detector through improving the coupling method. One is to use a higher ratio of human gamma globulin to goat anti-HSA pAb in this case. The resulting complex will have more human gamma globulin moiety that would be easily detected by an anti-human Ab detector. Another is to reduce the challenge ratio of cross-linker to both components allowing each molecule of one component only to link with one molecule of the other component. This would reduce the population of multimers in the hybrid complex and improve accessibility of the hybrid complex to antigens. A third method is to use a fraction of human gamma globulin, such as Fc fragment. As Fc fragment is smaller compared with whole

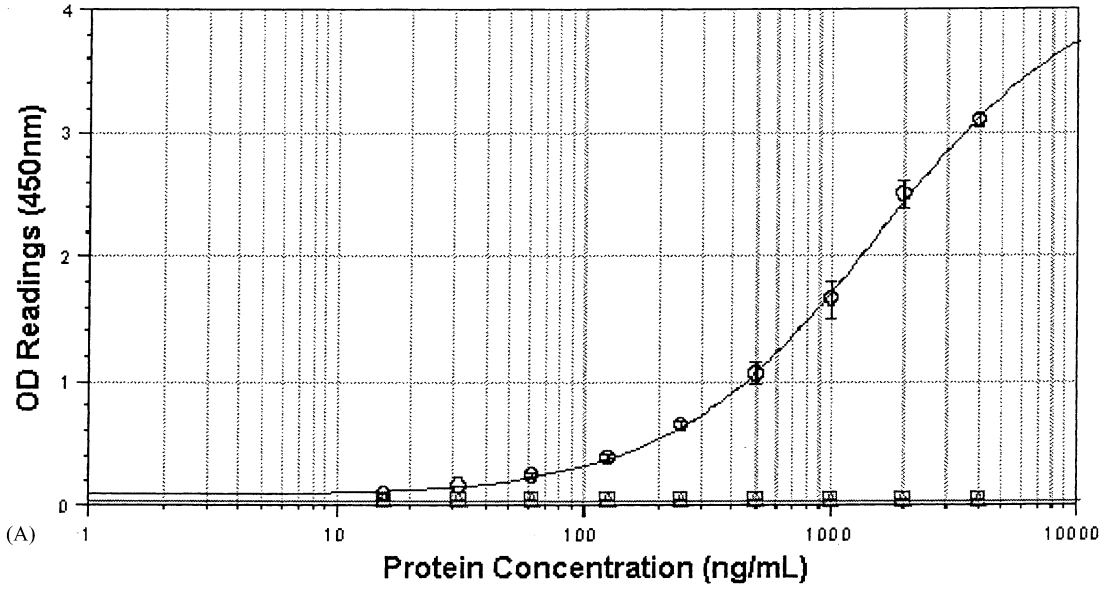

Fig. 3. Detection of the hybrid complex binding to the HSA coated plate (A). A microplate was coated with HSA. Goat anti-HSA pAb (△), human gamma globulin (□) or the hybrid complex (○) starting from 4 μg/ml were incubated with the plate at various concentrations. HRP-labeled goat anti-human conjugate was used to detect antibodies bound to the plate. Color conversion of TMB substrate was measured at 450 nm and signals were analyzed by Softmax program. Detection of goat anti-HSA pAb and the hybrid complex binding to the HSA coated plate (B). The assay condition was the same as described except goat anti-HSA pAb curve started from 0.5 μg/ml and HRP labeled rabbit anti-goat conjugate was used to detect antibody bound to the plate. The results were reproducible in three independent experiments with percentage CV less than 10 and are presented as mean ± S.D.

antibody, the resulting hybrid complex would experience less steric hindrance.

4. Conclusion

The method described in this paper demonstrates that a cross-linked antibody complex, composed of human gamma globulin attached to a specific animal anti-human protein, has the immunological appearance of a human antibody while being specific for binding to human antigens. This hybrid complex has value for use as positive control in ELISA for screening human clinical samples. This method is straightforward, and allows the creation of an array of hybrid complexes with a short period of time and low cost. When compared with the original specific antibody, some loss of activity is produced by the coupling reaction. However, the shift in binding curves is reasonable, and does not greatly impact the use of hybrid complexes as positive controls. Further optimization of the cross-linking chemistry might lead to a greater ratio of dimmer to multimers and further reduce the loss in activity observed. However, because cross-linking will depend on the antibodies used, each condition needs to be optimized individually. In ELISA, the hybrid complex generated was indistinguishable from any real human antibody in terms of antigen binding and secondary antibody detection. Another benefit of this method that it can apply to all kinds of antibodies from many animal species. In this lab, hybrid complexes, made by a mouse anti-human erythropoietin mAb and human IgG Fc fraction, and a mouse anti-human interleukin-2 mAb and human gamma globulin, were also generated for possible use as positive controls in ELISAs (data not shown).

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