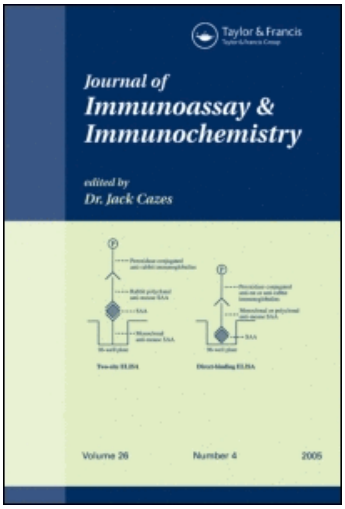

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Abstract: Serum autoantibodies to visual arrestin, also termed S-antigen, have been shown to accompany several autoimmune-related diseases. However, they were also detected in sera of healthy individuals; there is lack of a sensitive and fast method for evaluation of putative differences between those two groups of antibodies. We show that, using biosensor technology based on surface plasmon resonance (SPR), it was possible to characterize real-time interactions of immune sera with immobilized arrestin. Binding characteristics revealed different interaction kinetics of antiarrestin antibodies present in two distinct rabbit sera and, thus, broadened results of immunoblotting analysis. Therefore, we suggest that SPR-based biosensor technology might be a valuable method for monitoring and evaluation of antiarrestin antibodies in patients' sera.

Keywords: Arrestin, S-antigen, Antibody, Surface plasmon resonance

INTRODUCTION

Visual arrestin, largely known as S-antigen, is a 48-kD cytosolic protein which plays an important role in the process of visual signal transduction (phototransduction).[1] It is a highly immunogenic protein, inducing experimental autoimmune uveitis.[2] Autoantibodies to arrestin have been detected in

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the sera of patients with various diseases.[3–6] However, their incidence was often similar in sera of healthy subjects and it was difficult to prove that a relationship existed between a particular disease and the occurrence of antiarrestin antibodies.[4,7–9] Therefore, it was of special interest, not only to detect these antibodies in serum samples, but also to analyze their interaction with arrestin. The optical biosensor technology based on the phenomenon of surface plasmon resonance (SPR) allows real-time measurement of interaction between immobilized protein and its soluble ligand.[10] It has been proven to be a valuable tool in studies of macromolecular interactions, including those between antibodies and antigens.[11,12] Due to its sensitivity, it is able to detect even low affinity antibodies which are not visible with other detection systems. Several recent studies have confirmed the usefulness of SPR-based systems for detection and characterization of antibodies directly in sera and other body fluids.[13–16] We demonstrate that, using the SPR-based BIAcore system with bovine arrestin immobilized to the sensor chip CM5, one is able to detect antiarrestin antibodies in immune sera and to measure their interactions with the antigen in real-time.

EXPERIMENTAL

Materials

Biosensor chips, CM5, were from Biacore AB (Uppsala, Sweden). N-hydroxysuccinimide (NHS), acrylamide, bis-acrylamide (N,N′-methylene-bis-acrylamide), ammonium persulfate, sodium dodecyl sulfate (SDS), N,N,N′,N′-tetramethylethylenediamine (TEMED), and bromophenol blue were purchased from Fluka (Buchs, Germany). Nitrocellulose was from Schleicher & Schuell (Dassel, Germany). Ethanolamine was from Merck (Germany). CNBr-activated Sepharose 4B, Heparin-Sepharose, HiTrap™ Protein A affinity columns were from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Mouse monoclonal antibody, specific to visual arrestin, was a generous gift from dr. K. Palczewski, University of Washington. Alkaline phosphatase-conjugated antibodies to mouse and rabbit IgG, as well as BCIP/NBT color development substrate, were purchased from Promega (Madison, WI, USA). Aprotinin, Hepes, leupeptin, N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), pepstatin A, phenylmethylsulphonylfluoride (PMSF), soybean trypsin inhibitor, and other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA).

Purification of Arrestins

Native arrestins were obtained from stocks of bovine and human retinas stored in the darkness at -20°C. Retinas were homogenized at dim red light at 4°C in

a low ionic strength buffer (10 mM Hepes, pH 7.5) containing inhibitors of proteases (1 mM benzamidine, 1 mM PMSF, 1 μg/mL pepstatin, 1 μg/mL aprotinin, 20 μg/mL leupeptin). The homogenate was centrifuged at 44,000 × g for 50 min at 4°C and a supernatant containing soluble proteins (the retinal extract) was further used for purification of arrestin. Bovine and human arrestins were purified from retinal extracts using combined ion-exchange (DEAE cellulose) and affinity (Heparin-Sepharose) chromatographies according to the procedure described earlier.[5] Purities of isolated proteins were examined using SDS-PAGE and their identities were confirmed by immunostaining with monoclonal antibody specific to visual arrestin.

Antiarrestin Sera and Purification of Antibodies

Sera 885 and 886 were raised in rabbits by intradermal injection of 0.25 mg purified bovine arrestin in Freund's complete adjuvant per animal followed, after 4 weeks, by three booster injections of the same amount of antigen in Freund's incomplete adjuvant. IgG antibodies were purified from resulting sera using protein A HiTrap™ affinity columns, according to the manufacturer's recommendations. SDS-PAGE and immunoblot analysis confirmed that affinity-purified immunoglobulins belonged to the IgG class and were not contaminated with other proteins.

SDS-PAGE and Immunoblotting

Samples containing retinal extracts (10 μg of soluble proteins) or purified arrestins (0.5 μg) were resolved in SDS-PAGE using 12.5% polyacrylamide gels and transferred onto nitrocellulose membranes using the Hoefer Transphor TE22 system (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The membranes were blocked with 1% casein, incubated with analyzed sera or purified antibodies, and then with secondary (anti-mouse or anti-rabbit IgG) antibodies conjugated to alkaline phosphatase. Bound antibodies were visualized using NBT/BCIP-based substrate.

Surface Plasmon Resonance (SPR) Analysis

All experiments were performed using a BIAcore 1000 instrument (BIAcore AB, Sweden) and carboxylated dextran sensor chips CM5. All solutions were filtered (0.22 μm Millipore) and degassed before use. Purified proteins were immobilized on the sensor chip surface via amine groups using a standard procedure.[17] Briefly, the matrix of the chip was activated by injecting 35 μL of a 1 : 1 (vol/vol) mixture of 50 mM NHS and 200 mM EDC for

7 min at a flow rate of 5 μL/min. Arrestins diluted in 10 mM acetate buffer, pH 4.8, were injected into flowcells (channels) at concentrations of 40 μg/mL and coupled for 7 min at flow rate of 5 μL/min. One channel of the sensor was always kept free of protein solution and was further used as a reference (control).[16] After immobilization, the remaining reactive groups on the chip's surfaces were blocked by a pulse of 35 μL 1 M ethanolamine, pH 8.5. Changes of resonance units (RU) after completion of the immobilization were 1800 RU for human arrestin and 1600 RU for bovine arrestin, which corresponded to 0.376 pmol/mm^2 (2.16 ng/channel) and 0.334 pmol/mm^2 (1.92 ng/channel) of immobilized proteins, respectively.

Sera were diluted 1:200 in HBS-buffer (10 mM Hepes, pH, 7.4, 0.15 M NaCl, 3.4 mM EDTA, and 0.05% Tween 20) while the purified antibodies were diluted with 25 mM Tris/HCl, pH 7.4, containing 50 mM NaCl and used at indicated concentrations. Analyzed samples were injected into the control and the arrestin-coated channels of the CM5 sensor chip at 10–30 μL/min for 6–8 min. After each cycle, the sensor chip surface was regenerated by injecting 150 μL of 100 mM glycine/HCl, pH 2.0, at a flow rate of 30 μL/min. To compare antibodies from different sera, it was assumed, for simplicity, that all IgG immunoglobulins purified from each serum were able to interact with arrestin. Based on such an assumption, it could be further considered that antibodies purified from different sera were applied to the biosensor at the same concentration. Only then, was the estimation and comparison of averaged kinetic constants possible. Association (k_{on}) and dissociation (k_{off}) kinetic constants were calculated by BIAevaluation 3.1 software using a 1 : 1 Langmuir model and assuming that fitting to data is satisfied when $\chi^2 < 2$.

RESULTS

Evaluation of Rabbit Antiarrestin Sera

The quality of purified arrestins was analyzed by means of SDS-PAGE and immunostaining (Fig. 1A). Only one protein band corresponding to an apparent molecular weight of 48 kDa was stained in lanes containing purified bovine and human arrestins and these bands were specifically recognized by the antiarrestin monoclonal antibody. This indicated that both arrestins were of high purity and were suitable for further studies. Purified bovine arrestin was then used for the immunization of rabbits. In order to evaluate the reactivity and specificity of obtained sera, they were subjected to immunoblot analysis (Fig. 1B). The reaction was highly specific and sera recognized only one band in bovine and human retinal extracts and exhibited strong reactivity with purified arrestins. Although both antiarrestin sera recognized arrestin, one of them (886) showed markedly higher reactivity than the other one (885). To characterize these differences in terms of

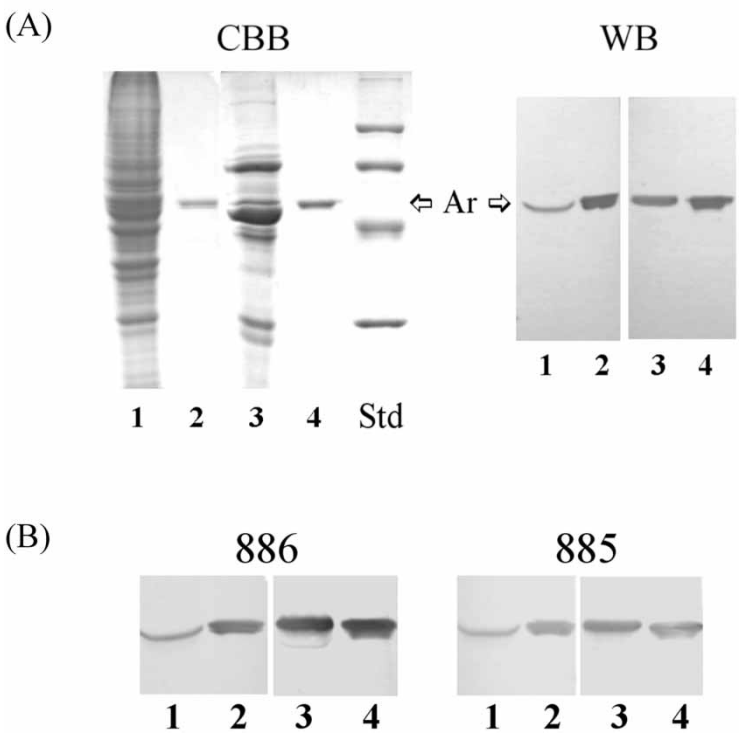

Figure 1. Evaluation of purified arrestins and rabbit antiarrestin sera. Retinal extracts (10 μg) and purified arrestins (1.0 μg) were resolved in SDS-PAGE using 12.5% polia-crylamide gels and subjected to Western blotting. Lanes 1–4 contained human retinal extract, purified human arrestin, bovine retinal extract, and purified bovine arrestin, respectively. (A), to evaluate purity of arrestins, the gels were stained with Coomasie brilliant blue (CBB) and blots (WB) were immunostained with mouse monoclonal antiarrestin antibody diluted 1:10,000. Molecular mass standards (Std) were: phos-phorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa). (B), to evaluate the specificity of antiarrestin antibodies in rabbit immune sera, blots were probed with serum 886 diluted 1 : 10,000 and with serum 885 diluted 1 : 5,000. Other details are described in Experimental section.

real-time binding, in the next step, both sera were injected to the CM5 sensor chip and their interactions with immobilized arrestins were compared.

Real-Time Interaction of Rabbit Sera and IgG with Bovine Arrestin

Interactions of antiarrestin sera 885 and 886 with immobilized bovine arrestin were detected in real time by SPR using a BIAcore system and resulting sen-sorgrams were recorded (Fig. 2). Response of the biosensor to serum 886 was

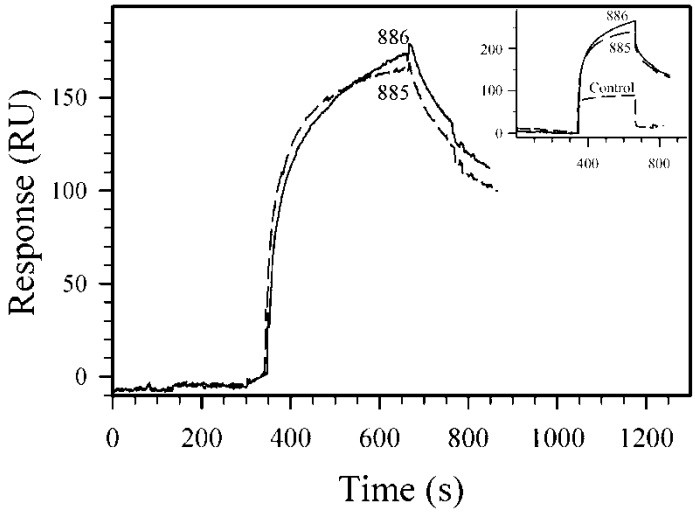

Figure 2. Binding of antiarrestin sera to sensor chip with immobilized arrestin. Rabbit sera 885 and 886 were diluted 1 : 200 with HBS and injected to CM5 sensor chip flowcell (channel) with immobilized bovine arrestin. Overlay of corrected sensorgrams obtained for each serum as a difference between arrestin-loaded channel and control channel is shown. Inset shows not corrected sensorgrams for each serum and control sensorgram which represents binding to the arrestin-free flowcell.

higher than it was to serum 885. Corresponding sensorgrams differed in the association phase and showed similar shape during dissociation of formed complexes. The interaction of sera with control channel was weaker, giving a considerably lower signal (inset in Fig. 2). To test whether the distinct response of the biosensor to each serum resulted indeed from binding of anti-arrestin antibodies, the IgG antibodies were affinity purified from each serum and subjected to analysis (Fig. 3). Response of the biosensor was dose-dependent (inset in Fig. 3). Resulting sensorgrams showed that this time response of biosensor was also higher to antibodies isolated from serum 886 (IgG886) and the difference was much more evident than was observed in the case of sera (Fig. 2). Interactions of purified antibodies with immobilized arrestin again differed in their association phase and exhibited similar shape of the dissociation. Estimated association rate of IgG886 ($4 \times 10^4\ M^{-1}s^{-1}$) was about two-fold higher than that of IgG885, while their dissociation rates were in the same range of $1.5 - 1.9 \times 10^{-3}\ s^{-1}$.

Interaction of Purified IgG with Bovine and Human Arrestins

Bovine and human arrestin share about 80% of amino acid sequence; we asked whether S-antigens from different sources could be equally useful in assays.

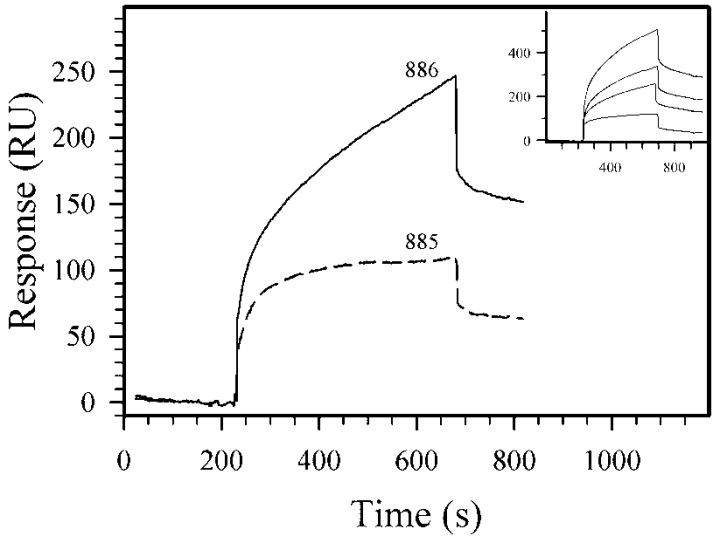

Figure 3. Interaction of antiarrestin IgG with immobilized arrestin. Rabbit IgG antibodies were affinity purified from sera 885 and 886 and injected at concentrations of 150 μg/mL onto CM5 sensor chip with immobilized bovine arrestin. Overlay of corrected sensorgrams for interaction of IgG purified from each serum is shown. Inset shows an overlay plot of sensorgrams obtained for interaction of bovine arrestin with purified IgG 886 applied at increasing concentrations 50, 100, 150, and 300 μg/mL.

Both arrestins were immobilized on the chip surface and their interactions with antiarrestin antibodies were compared (Fig. 4). A higher response of the biosensor was noted in the case of bovine arrestin. This could be, at least in part, due to its higher surface density, but estimated kinetic constants were also different. The association rate was slightly higher and the dissociation rate was about two-fold lower than corresponding values estimated for the interaction with human arrestin. These differences were in line with results of immunoblot analysis (Fig. 1B).

DISCUSSION

The aim of this study was to adopt a method convenient for evaluation of serum antibodies to S-antigen. Two antiarrestin rabbit sera, which exhibited different reactivities in immunoblotting, were used as a model. It was not possible to determine the affinity of antiarrestin antibodies in these polyclonal sera because of unknown amounts of antibodies of different classes and specificities. To characterize their reactivity with arrestin, a BIAcore system was employed. Systems based on the SPR technology have been already applied to

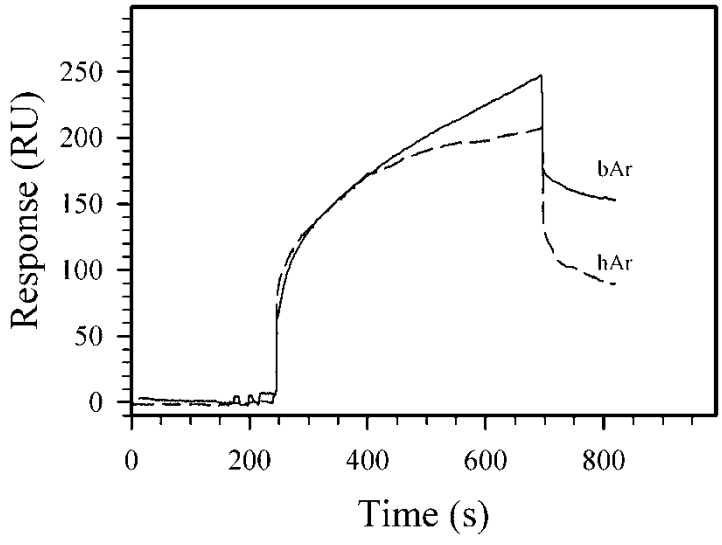

Figure 4. Interaction of antiarrestin antibodies 886 with bovine and human arrestin. Antiarrestin IgG antibodies, purified from rabbit serum 886 as described in Experimental, were injected at concentration of 150 μg/mL onto CM5 sensor chip immobilized with bovine (bAr) and human (hAr) arrestins. Overlay of corrected sensorgrams obtained for interaction of IgG886 with each arrestin is shown.

the detection of antibodies in sera samples.[14–16] The most advantageous aspect of this type of assays is that antibodies to a particular antigen can be detected in real time directly in serum samples; very small quantitities of material are needed.[11,12] Another important feature is that corresponding sensorgrams reflect averaged kinetics of the association and dissociation of all serum components interacting with an immobilized protein.[12] Thus, using SPR-based assay, we could compare overall interactions of two polyclonal sera with arrestin immobilized to the sensor chip. The results obtained confirmed higher reactivity of serum 886. The difference became more evident when affinity-purified IgG antibodies were injected into the biosensor. This observation suggested the presence in sera also of antibodies of other classes, possibly IgM, which were lost during the purification procedure. Such an explanation was consistent with the results of immunoblotting, where only IgG antibodies bound to arrestin were detected. We have also attempted to determine whether different interactions of IgG886 and IgG885 with arrestin could be explained in terms of binding constants. The assumption that all IgG immunoglobulins purified from each serum possessed antiarrestin reactivity enabled us to estimate apparent kinetic constants of interactions. The calculated values of dissociation rates for IgG885 and IgG886 were similar but IgG886 revealed a significantly higher value of the association constant, indicating their higher affinity. However,

it should be pointed out that the shape of sensorgram indicated also possible heterogeneity of the IgG886 binding to immobilized arrestin. It could not be determined precisely whether the calculated higher association rate resulted from the higher affinity of antiarrestin antibodies or from their higher real quantity in the IgG fraction. Another important observation was that rabbit antibodies to bovine arrestin also recognized human arrestin, as proven by the immunoblot and the SPR analysis. Their different interactions with each arrestin indicated that at least some recognized epitopes had different sequences in human and bovine arrestins. Therefore, one should use arrestin isolated from species different than analyzed sera with caution.

In summary, this study established that purified arrestins (S-antigens) immobilized on the surface of a biosensor could be used for the detection of antiarrestin antibodies in immune sera by means of the SPR analysis. It also proved that the method was applicable for the characterization of their interactions by analysis of the corresponding binding curves. It might be especially useful for monitoring of the appearance and evaluation of antiarrestin antibodies in sera of patients with autoimmune diseases in comparison with those detected in sera of healthy subjects.

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