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The First Aptamer-Apheresis Column Specifically for Clearing Blood of β1-Receptor Autoantibodies

– A Successful Proof of Principle Using Autoantibody-Positive SHR Rats –

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Background: Application of immunoapheresis to eliminate pathogenic autoantibodies targeting the second extracellular loop of the β1-receptor (β1-AABs) is currently investigated in patients with cardiomyopathy. Aptamers (single short DNA or RNA strands) are a new class of molecules that bind to a specific target molecule. This property qualifies aptamers for potential use in the apheresis technique. We recently identified an aptamer that specifically binds to β1-AABs, so in the present study we tested whether this aptamer could be used as a binder to prepare an apheresis column suitable for clearing β1-AABs from rat's blood.

Methods and Results: An apheresis column was designed containing the β1-AAB-targeting-aptamer coupled to sepharose. As tested in vitro, this column (1) binds β1-AABs highly specifically without marked interference with common IgGs, (2) has a capacity for clearing of approximately 1L of β1-AAB-positive serum and (3) can be completely regenerated for subsequent use. Using the column for extracorporeal apheresis of spontaneously hypertensive rats (SHR) positive for both β1-AABs and muscarinic 2-receptor autoantibodies (M2-AABs), only β1-AABs were removed. In a follow-up of 9 weeks, recurrence of β1-AABs in the blood of SHR could not be detected.

Conclusions: For the first time, a newly designed apheresis column with a β1-AAB specific aptamer as a binder was successfully used to eliminate β1-AABs from SHR blood.

Key Words: Apheresis; Aptamer; Autoantibody; Dilated cardiomyopathy; Immunoadsorption

We present here for the first time in vitro and animal experiments demonstrating aptamer-based clearing from blood of pathogenic autoantibodies targeting the second extracellular loop of the β1-receptor using apheresis technology.

We recently published the selection and identification of a novel ssDNA aptamer that targets autoantibodies directed against the second extracellular loop of the adrenergic β1-receptor (β1-AABs).[1] As recently summarized,[2,3] autoantibodies directed against the first or second extracellular loop of the adrenergic β1-receptor (ie, β1-AABs) have been found in patients with cardiomyopathies, with evidence that mainly β1-AABs directed at the second loop are related to disease pathogenesis.[4] Up to 80% of patients with dilated cardiomyopathy (DCM) are positive for either first- or second-loop β1-AABs; approximately one-half of the patients present with first-loop β1-AABs, the other half with second-loop β1-AABs. As demonstrated in a Bolivian cohort, nearly 100% of patients with Chagas' cardiomyopathy are positive for second-loop β1-AABs.[5] A first indication exists that exclusively second-loop β1-AABs were found also in patients with peripartum cardiomyopathy.[6]

Consequently, technologies have been developed to clear β1-AABs from the blood of β1-AAB-positive DCM patients via immunoapheresis. In addition, β1-AAB immunoapheresis has been suggested for the treatment of Chagas' patients.[7] Col-

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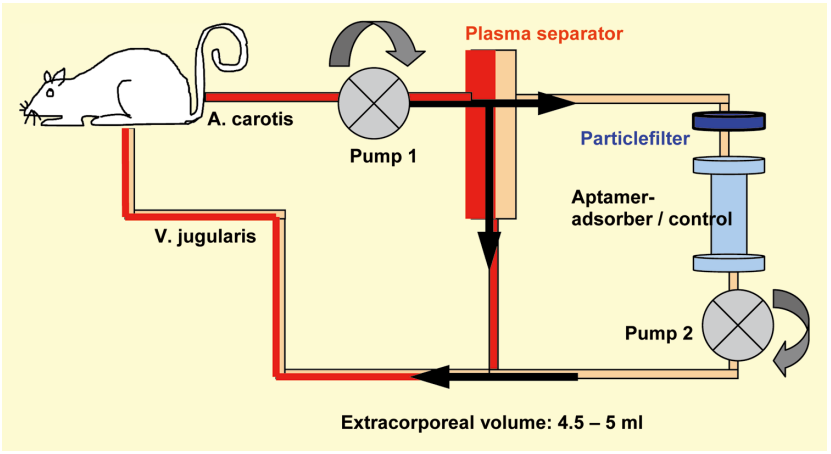

Figure 1. Schematic illustration of the apheresis procedure.

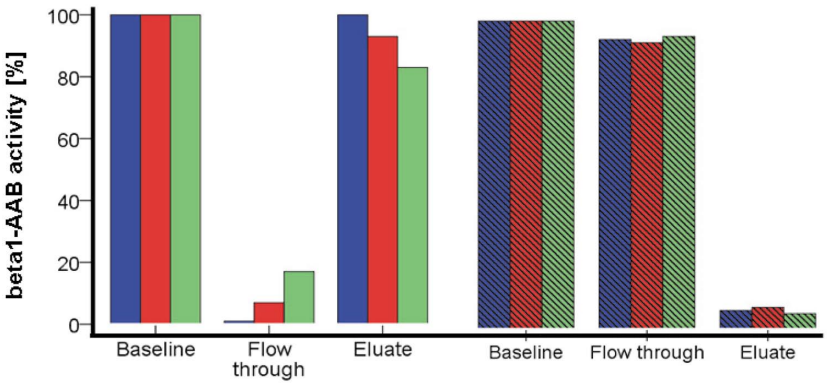

Figure 2. In vitro AAB activities measured via bioassay in the original samples, column flow-through, and column eluates. (**Left**) Application of specific truncated aptamer 110-sepharose column. (**Right**: shaded bars) Application of control apheresis column (ethanolamine-inactivated sepharose). Blue bars: serum sample from a β1-AAB-positive DCM patient. Red bars: IgG-fraction of DCM patient's material. Green bars: goat anti-human β1-receptor spiked serum samples. AAB, autoantibodies; DCM, dilated cardiomyopathy; Ig, immunoglobulin.

umns that bind the entire IgG fraction, thereby clearing β1-AABs, are currently used in clinical studies.[8] To minimize these patients' infection risk because of the severe IgG loss, post-apheresis IgG substitution is recommended.[9] Other technologies use peptides that specifically bind β1-AABs. In both technologies of β1-AAB clearance, the benefit to patients is well documented and, as recently published, can persist for over 12 years after the initial apheresis.[10] Showing the equal outcome of both technologies is, moreover, an additional clear indication for the leading role of β1-AABs in DCM pathogenesis. A therapeutic benefit also holds true for Immunsorba TR selective IgG3 columns, as recently investigated even under blinded measurement conditions.[11]

Our previously selected aptamer using the Monolex technology[12] bound β1-AABs isolated from patients with DCM, Chagas' cardiomyopathy, or peripartum cardiomyopathy and neutralized their pathogenic functions, which we demonstrated in vitro by reducing β1-AAB-induced chronotropy and apoptosis.[1] However, as recently discussed,[13] the relationship between the selected aptamer and β1-AABs must be consolidated in vivo using animal models.

Therefore, we initiated a first line of animal experiments in order to demonstrate the aptamer's potency for in-vivo disease treatment. For this purpose, we designed an aptamer 110-coupled apheresis column, and tested its ability to clear these antibodies from the blood of β1-AAB-positive rats. After in vitro testing of the functionality of the apheresis column, we subjected rats that were positive for β1-AABs and, in addition, for autoantibodies against the muscarinic 2-receptor (M2-AABs), to an extracorporeal apheresis treatment. To qualify the success of the apheresis, we measured the rats' autoantibody blood titers before the apheresis, immediately after, and also days/weeks later.

Methods

Materials and Animals

Sera of patients with DCM and positive for β1-AABs were obtained from Deutsches Herzzentrum Berlin. The use of these sera for in vitro experiments, and of IgGs prepared from the sera, were approved by the hospital's authorities. All patients signed an informed consent form. Spontaneously hypertensive male rats (SHR/FubRkb, SHR, RGD:631696) bred at the Forschungseinrichtung für Experimentelle Medizin at Charité-Universitätsmedizin Berlin were treated under animal experiment license number: 7221.3-1,1-006/11, and tested to be positive for β1-AABs. Two of the animals were also positive for M2-AABs. At the beginning of the experiments, the mean body mass of the animals with an age of ≈18 months was 393±27 g. All animals' hematocrits were >0.40 L/L to guarantee that any extracorporeal apheresis-associated hematocrit lowering, which can be up to 20%, did not interfere with the study protocol.

Apheresis Column Preparation

For the preparation of the apheresis column, with respect to its technological aspects, the aptamer 110, which was originally a 63 mer, was processed by truncation to identify the minimal sequence comparable to the full length nucleotide sequence in its interaction with β1-AABs. A 12 mer aptamer (GCG TGA GGT CGA) was identified as being equivalent in functionality to the original aptamer 110. The aminohexyl-modified truncated sequence (synthesis, purification and coupling performed by BioTez Berlin-Buch GmbH, Berlin, Germany) was covalently coupled to NHS-activated sepharose (NHS-activated Sepharose 4 fast flow, 17-0906-01, from GE Healthcare Europe, Munich, Germany) and used to fill a 3-ml SPE column (NeoLab) for in vitro testing, as well as a Tricorn 10/20 column (28-4064-13, GE Healthcare Europe) for in vivo application. The capacities of the columns were 0.1 μmol coupled aptamer for the SPE column, and 1.1 μmol coupled aptamer for the Tricorn 10/20 column. As a control, ethanolamine-inactivated sepharose 4 fast-flow columns were used.

Extracorporeal System for Plasmapheresis in Conscious Rats

The technical equipment and procedure described here were based on methodologies published in 1980,[14] and were tested on unrestrained, non-anesthetized, male rats with a body weight of 200–500 g.

Animal Handling

Chronic catheters (PhysioCath, DSI, St. Paul, MN, USA) were inserted under general anesthesia (i.p. injection of 0.8 mg Rompun®, Bayer AG, Leverkusen, Germany and 4 mg Ketamin®, Sanofi-Aventis, Berlin, Germany). Each animal was placed on its back, an incision was made in the neck region, and the carotid artery and jugular vein were dissected. The blood flow of each vessel was interrupted with 1 fixed ligature cranial and 1 open ligature caudal. Catheters were then introduced into the respective vessels, followed by fixation of the caudal ligature, and fixed in the vessels with an additional ligature for stabilisation. To exteriorize the catheter, a small incision was made in the dorsal nape. The catheters were tunneled subcutaneously from the insertion point to the exit in the nape, after which the incision in the nape was closed. The catheters were filled with a heparinized saline solution and locked with a stopper. They were, outside of the animals, useable at any time without stress to the rat. Following a convalescence period of approximately 7 days, the catheters were functional for 4–5 weeks.

Equipment

Prior to apheresis, the catheterized rats were sitting freely in a basket, and were adapted to human contact by daily handling for 4 days. There was only gentle, careful fixation of the animal during the entire apheresis procedure. As demonstrated schematically in **Figure 1**, the catheter ends were connected to the extracorporeal system, and blood was pumped (multichannel pump, Petro Gas Ausrüstungen, Berlin, Germany) from the animal's carotid artery to the plasma separator (Saxonia Medical, Radeberg, Germany) for separation of blood particles and plasma. The blood particles and the plasma, after passage through the aptamer-containing adsorber column, were then returned to the rat's jugular vein supported by a second pump.

Beta1- and M2-AAB Measurements

The AAB activity was measured using a bioassay for β1-AAB estimation.[15] Briefly, the AAB activity of the IgG-fraction from heparin-plasma obtained via an ammonium sulfate precipitation procedure was tested using spontaneously beating neonatal rat cardiomyocytes. After estimation of the basal beating rate of 6 independent and marked cell clusters, the IgG preparations were added to a final dilution of 1:40 and incubated for 60 min, before the same marked spots were used for a repeat estimation of the beat frequency. The change in frequency was expressed as Δ pulsation rate/min (ΔPR [1/min]). The β1-AABs caused an acceleration of the beating rate of the spontaneously beating cardiomyocytes. In contrast, the muscarinic M2-AABs induced a negative chronotropic effect. In order to exclude any interference by the other respective AABs, each measurement was carried out in the presence of an excess of specific blockers of the particular other AABs: bisopolol (1 μmol/L) to block the β-receptors and measure M2-AAB activity, and atropine (1 μmol/L) to block the muscarinic M2-receptor and measure the remaining β1-AAB activity.

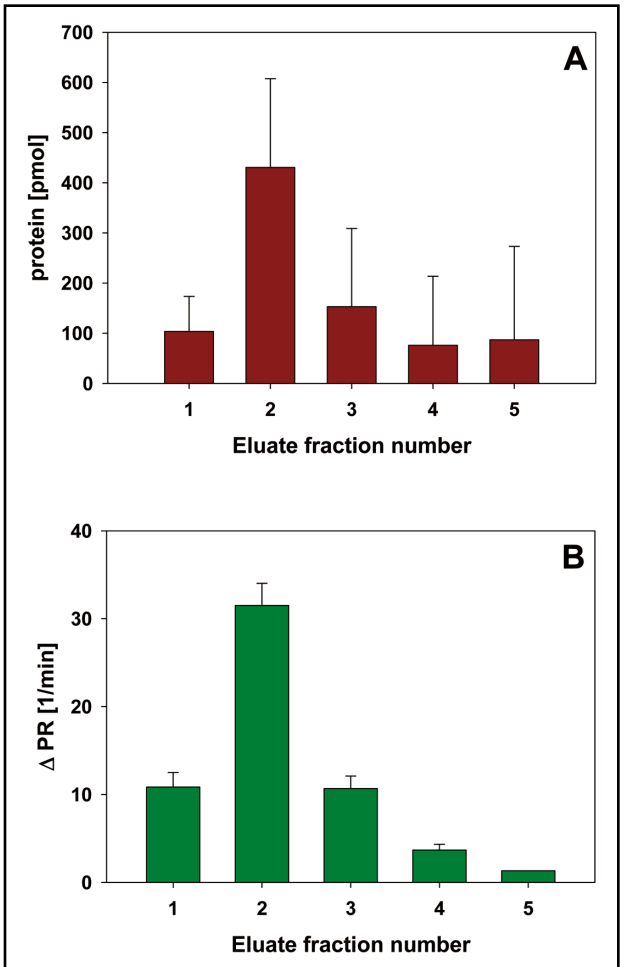

Figure 3. (**A**) Protein concentrations and (**B**) chronotropic AAB activities of single eluate fractions from in vitro testing of the performance of the specific aptamer column using IgG preparations. The bars represent the mean ± SD. of 4 independent IgG preparations from 4 different patients. ΔPR, change in pulsation rate (contractions/min). AAB, autoantibodies; Ig, immunoglobulin.

IgG Concentrations in Plasma and Eluates

To compare IgG concentrations in plasma before and after apheresis, and in the eluates, the following procedure was performed. The samples were diluted in stepwise increments

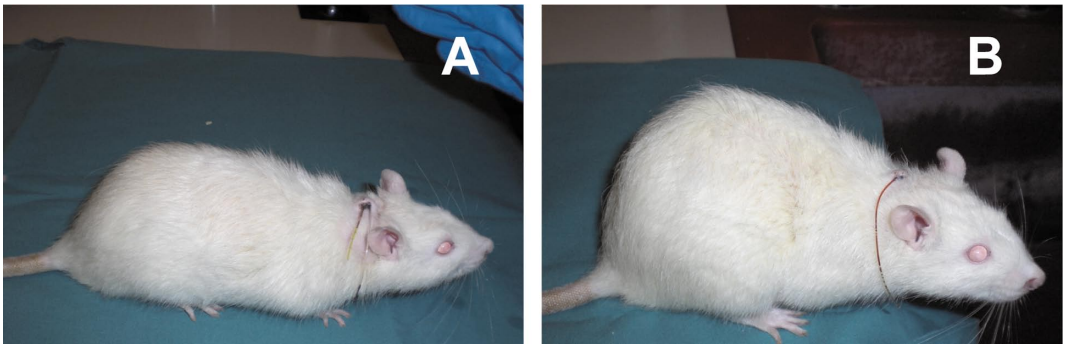

Figure 4. Appearance of (**A**) aptamer-apheresis-treated rat 11 days after the final apheresis and (**B**) control rat 12 days after apheresis.

of 1 order of magnitude each, starting with 1:200 up to 1:2,000,000. The samples were then immobilized on an ELISA plate (Maxisorb, Nunc, Thermo Fisher, Soeborg Denmark) using a 0.1 mol/L carbonate buffer pH 9.25; after incubation overnight at 4°C, the supernatants were removed, and the plates were washed and blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS) before the adsorbed IgG was quantified using an anti-rat IgG (H+L)-HRP secondary antibody (Dianova, Hamburg Germany) and the H_2O_2/TMB detection system (measuring at E 450 nm, MRX Microplate Reader, Dynex Technologies, Chantilly, VA, USA). The slopes of the resulting curves were then compared.

Measurements of Protein Concentration in the Single Eluate Fractions

The protein concentration in each fraction was estimated by measuring the optical density at 280 nm (Shimadzu UV-2101PC, UV-Vis Scanning Spectrophotometer, Shimadzu Deutschland, Duisburg, Germany), and calculating the amount of IgG using a factor of 1.35.[16]

In Vitro Testing of Apheresis Column Functionality for β1-AAB Clearance

For in vitro testing of the column's functionality and performance, the following samples were applied: (1) sera of patients with DCM and positive for β1-AABs, (2) IgGs prepared from serum aliquots and diluted in PBS-buffered NaCl to an IgG concentration comparable to that of the primary serum, or obtained from the eluate from the apheresis treatment, and (3) β1-AAB-spiked human serum prepared using the commercially available antibody ADRB1 (goat anti-human antibody against the second loop of the human adrenergic β1-receptor, EB07133, Everest Biotech, Oxfordshire, UK). The spiked β1-AAB activity was comparable to the patient's serum β1-AAB activity.

Next, 250 µl of each of the 3 preparations were passed through both the apheresis column and the control column. The columns were then washed using PBS, and eluted 3 times using 250 µl of 3 mol/L KSCN or 6 mol/L NaCl per fraction. Both the column flow-through and eluates were analyzed for AAB activity.

Animal Experiment

At 7 days after catheter implantation, the rats were attached to the apheresis equipment as demonstrated schematically in Figure 1. Apheresis treatments with the aptamer-coupled column were performed repeatedly, 4 times in a row (1 treatment per day for 4 successive days) with 2 rats, and twice with 1 rat, to fully clear the animals' plasma in each session (1 session equaled 1 apheresis cycle). Using the same procedure, 1 rat was treated using the control column. The treatment protocol was adapted to procedures that are used in clinics for human apheresis. After each apheresis, the column was eluted with 5 fractions of 0.5 ml of 6 mol/L NaCl, which were then dialyzed against isotonic buffer and measured for β1- and M2-AAB activity. After finishing the apheresis, each rat's plasma was analyzed for the presence of β1- and M2-AABs, a measurement that was repeated 2 and 9 weeks after apheresis, and again after humanely killing the animals at either 133 (2 animals) or 84 days (1 animal) after apheresis. The different end-points are because the apheresis was performed on different days, but the end-point of the experiment was the same for all animals.

Results

In Vitro-Testing of Column Functionality

To evaluate the specificity of the aptamer column, aptamer-based and control columns were tested for their abilities to trap β1-AABs from (1) DCM serum; (2) IgG preparation and (3) β1-AAB (ADRB1) spiked control serum. The β1-AAB concentration in the flow-through material of the control column was nearly comparable to the original serum AAB concentration. With the aptamer column, a strong reduction in β1-AABs was seen. After column elution, no β1-AABs were found in the eluates from the control column, but the eluates from the aptamer column contained β1-AABs in quantities comparable to the original material (Figure 2).

The specific data for the aptamer vs. control columns were as follows: (1) DCM serum: flow-through=–1.32 vs. 20.68 ΔPR/min, eluate=25.32 vs. 1.32 ΔPR/min; (2) IgG preparation: flow-through=1.34±0.93 vs. 20.0 ΔPR/min, eluate=23.0± 1.41 vs. 4.68 ΔPR/min (n=2 for the aptamer column); (3) ADRB1-spiked serum: flow-through=5.32 vs. 25.32 ΔPR/min, eluate: 21.32 vs. 1.32 ΔPR/min.

Based on the aptamer–sepharose coupling scheme described in the Methods, the theoretical capacity for binding 0.1 µmol β1-AAB with the SPE column, and for 1.1 µmol with the Tricorn 10/20 column was calculated. Assuming a concentration of β1-AABs in plasma of 0.1% of the total IgG, these binding capacities for β1-AABs should be adequate to clear nearly 100

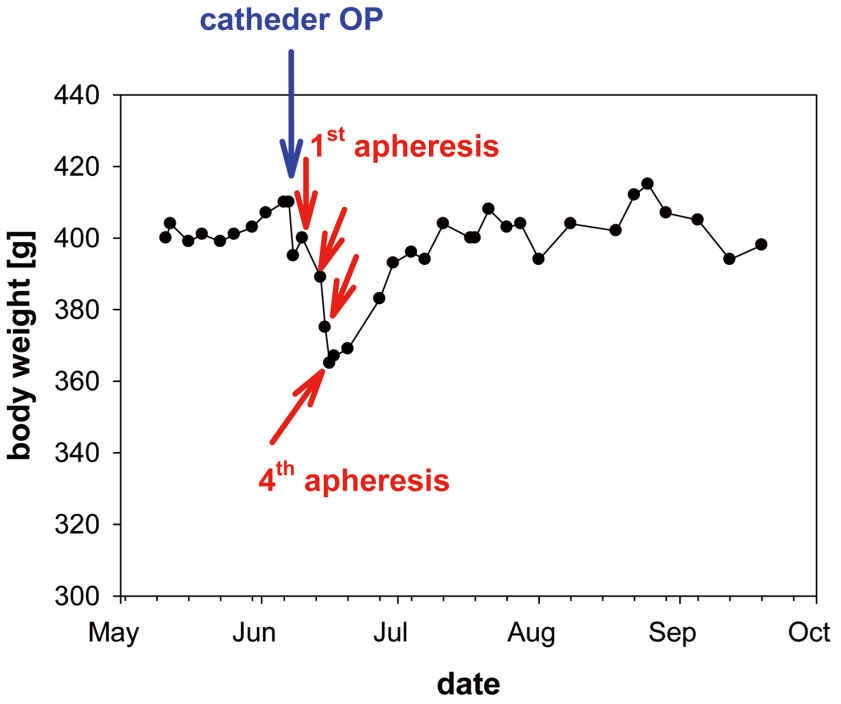

Figure 5. Physiological condition of rats during the experiment. A representative monitoring curve of the body weight of 1 aptamer-apheresis-treated rat throughout the whole experiment.

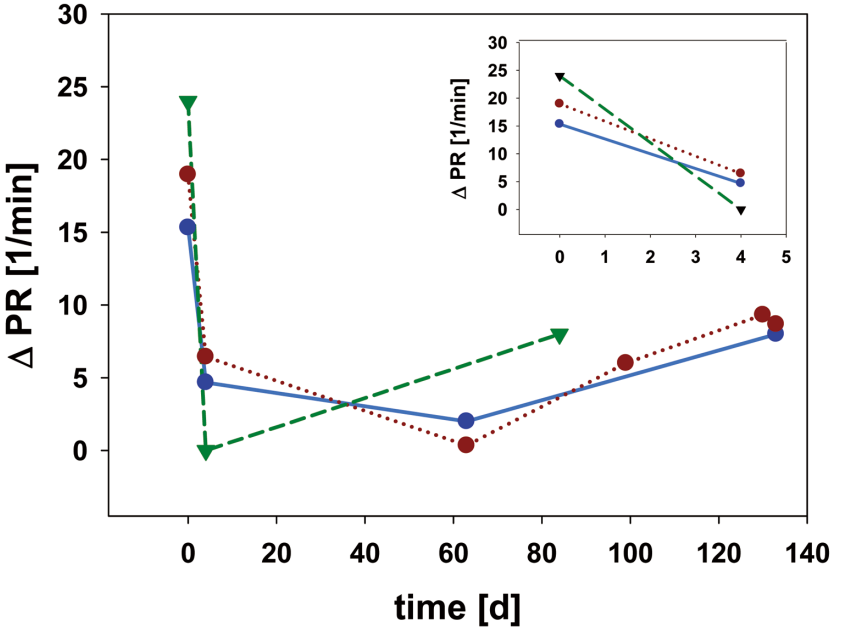

Figure 6. Beta1-AAB titers throughout the experiment. Day 0, before apheresis; day 4, after completion of all apheresis cycles. For better demonstration of day 0 (before apheresis) and day 4 (after completion of all apheresis cycles), values have been additionally inserted in the extra window in the upper righthand corner of the graph. Blue circle and green triangle for 4 apheresis cycles; brown circle for 2 apheresis cycles. The β1-AAB activity was estimated using the bioassay as indicated under Methods and is expressed as ΔPR (change in pulsation rate: contractions/min). AAB, autoantibodies.

and 1,000 ml of β1-AAB-containing plasma, respectively. Under our experimental conditions, only 1–5% of the column's binding capacity was used. In order to demonstrate that the residual binding capacity was not used for any nonspecific binding of serum proteins, especially of common IgGs, the eluate was sampled in fractions in which the β1-AABs and the protein concentration were measured in parallel (**Figures 3A,B**). Significantly more protein was not found in the eluate, calculated according to its content of β1-AABs, so the eluted protein was clearly associated with the β1-AABs, as demonstrated for the SPE column in **Figure 3**. This finding was a further experimental indication of the specificity of the aptamer column for β1-AABs.

Rat β1-AAB Apheresis

Basic Information No obvious phenotypic and behavioral

changes of the rats were seen during the acute stage of the experiments, starting with catheter implantation to the cessation of the apheresis procedure. The experiments with the SHR were started when they were ≈18 months old, an age that ensured a high autoantibody titer. The appearance of the fur of these animals was less smooth and soft as compared with young SHR (**Figure 4A**). Although subjective, after aptamer-column apheresis, the rats' fur appeared to be more smooth and soft for a couple of weeks before notably reverting to reflect their age. **Figure 4** shows control- and aptamer-apheresis treated rats with implanted catheters after apheresis treatment for comparison. The body weight of the rats did not change significantly during the single apheresis treatments. Looking at the complete procedure of apheresis treatment, taking into account the catheter implantation and the 4 or 2 consecutive apheresis cycles, the body weights decreased up to ≈10% of the initial weights but returned to baseline values after finishing the treatments. The results of body weight monitoring are demonstrated representatively for 1 rat in **Figure 5**.

AAB Activities After finishing the apheresis treatment regimen using the control column, the rat plasma IgG and AAB concentrations were nearly unchanged compared with the pretreatment values. In agreement with these results, almost no protein or IgG could be detected in the eluate from the control column (data not shown).

In contrast, although the total plasma IgG concentration did not change significantly, the β1-AAB concentration was strongly reduced in the plasma of rats treated with the apheresis column (before apheresis: 18.37±4.88 ΔPR/min, after apheresis: 4.89±5.00 ΔPR/min (n=3), $P<0.05$, t-test, 2-sided), whereas the plasma activity of M2-AABs did not change in the 2 rats testing positive for M2-activity (before apheresis: −15.34±0.93, after apheresis: −16.00±0.00 ΔPR/min (n=2) (**Figure 6**). Consequently, the eluates from the aptamer column presented with very low total IgG concentration (nearly 1/5,000 of the concentration of rat serum) whereas β1-AAB activity was found in quantities corresponding to the baseline rat plasma activity. No M2-AAB activity was detected in the eluate.

In the follow-up of plasma β1-AAB activity, the AABs remained low for at least 60 days before reaching approximately 50% of the baseline level only at the end of the experiment on days 84 and 133, respectively (**Figure 6**).

Discussion

We present here for the first time (1) the design and in vitro testing of an apheresis column using a carefully selected aptamer coupled to sepharose that was able to specifically remove β1-AABs, and (2) data from animal experiments demonstrating that this column could be used successfully in an apheresis application to clear β1-AABs from rat blood.

The value of apheresis to clear pathogenic β1-AABs from serum has already been proven[8,9,11] and the long-term benefit is undoubted.[10] One disadvantage with the application of established adsorbers is the fact that total immunoglobulins, or entire classes of immunoglobulins, are removed from the body, including the AABs. In the first case, follow-up supplementation with immunoglobulins is strongly recommended to reduce the risk of infection. In order to overcome this shortcoming, peptide adsorbers for β1-AABs, which specifically trap the AABs, were tested and found to be comparable in their benefit to patients.[10]

The idea of combining the advantages of aptamer technology with column chromatography in order to develop specific adsorbers was very challenging. Ruta et al developed a well-performing aptamer-modified column that efficiently enabled separation of an adenosine enantiomer,[17] while Connor and McGown exploited a specific aptamer for the development of a specific stationary phase in affinity capillary chromatography.[18] Hu et al also developed and tested the application of aptamer-adorbers for purification of drinking water from pharmaceuticals,[19] just to mention a few activities in this field. Still, so far not a single application has yet reached the commercial level. Similarly, with respect to medical applications of apheresis technology, no example has been reported, according to our best knowledge, although the technical advantages of aptamers, such as being autoclavable, should render them ideal for this purpose.

However, because of the absence of appropriate aptamers, until now no aptamer-based adsorber has been able to clear AABs from blood, particularly those that are pathogenetically active and directed to G-protein coupled receptors. Using our recently selected in-vitro characterized aptamer,[1] which targets β1-AABs found frequently in patients suffering from DCM, Chagas' cardiomyopathy or peripartum cardiomyopathy, we were able to design and test an aptamer-based adsorber specific for β1-AABs. To simplify the development of the column, the 63 mer aptamer 110[1] originally selected was shortened to a 12 mer, which still showed the same properties as the original 63 mer sequence. Successful in vitro pretesting of the aptamer-coupled column permitted advancement to in vivo testing.

The abundance of human β1-AABs in the IgG fraction has been calculated to be approximately 0.1% of the total, assuming that the β1-AABs behave similar to AABs against thyroid-stimulating hormone receptor in humans, for which a similar number has been estimated.[2] Because a similar composition might also be assumed in rats, we estimated our column for rat apheresis possessed a binding capacity for β1-AABs, based on the coupled aptamer concentration, sufficient for clearance of approximately 1,000 ml of serum. This means that only 1–5% of the column's capacity was used to clear the blood of the test rats.

The AABs in the eluates from the apheresis column were still functionally active, as shown using a bioassay. Although the β1-AABs were trapped by the aptamer column, this was not the case, as expected, for muscarinergic M2-AABs. Here the plasma retained full activity even after the 4th cycle of apheresis treatment.

Conclusions

In summary, an apheresis column based on a severely truncated aptamer 110 was prepared and tested for its performance in clearing β1-AABs from plasma. The exact binding mechanism between aptamer and AAB has not been identified yet. But, in an analogy to published aptamers specific for AABs against the insulin receptor[20] and the acetylcholine receptor,[21] a direct interaction with the epitope-binding region of the AABs might be assumed. The apheresis column was applied to clear the blood of rats positive for β1-AABs and M2-AABs. After the apheresis treatment, each rat's blood was strongly reduced in its β1-AAB concentration, whereas the total IgG concentration was not affected. This, in our view, represents a successful in-vivo proof of concept of the applicability of aptamer 110 in apheresis technology for the elimination of β1-AABs, in order to counteract their potency in the pathogenesis of DCM, Chagas' cardiomyopathy, and peripartum cardiomyopathy. However, to push this concept towards medical treat-

ment, long-term follow-up studies, including the outcome of animals treated with aptamer-based β1-AAB apheresis, are necessary.

Acknowledgments

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