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DARPins against a functional IgE epitope

Michael J. Baumann[a,b], Alexander Eggel[a], Patrick Amstutz[b], Beda M. Stadler[a], Monique Vogel[a,*]

[a] Institute of Immunology, University of Bern, Inselspital, Sahlihaus 2, 3010 Bern, Switzerland
[b] Molecular Partners AG, Wagistrasse 14, 8952 Zürich-Schlieren, Switzerland

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ABSTRACT

The monoclonal anti-IgE antibody omalizumab (Xolair®) is mostly used for the treatment of severe allergic asthma. However, the requirement of high doses and suboptimal cost-effectiveness limits the use of the treatment. Here we propose to use a new drug format based on non-immunoglobulin structures, potentially offering increased clinical efficacy while being more cost-effective. For this purpose, DARPins™ (designed ankyrin repeat proteins) against the constant heavy chain region of IgE have been isolated. DARPins were binding to IgE with high specificity and affinities in the low nanomolar range. Selected DARPins antagonized the interaction between IgE and its high-affinity receptor in inhibition assays. Furthermore, anti-IgE DARPins were shown to inhibit proinflammatory mediator release from rat basophilic leukemia cells expressing human high-affinity IgE receptors with higher efficacy than the monoclonal anti-IgE antibody omalizumab. DARPins may thus represent promising future drug candidates for the treatment of allergy.

1. Introduction

Since the identification of immunoglobulin E (IgE) as key mediator of immediate hypersensitivity in asthma and allergic disorders [1–4], controlling of the IgE response has become one of the main therapeutic objectives. Direct targeting of IgE interaction with its high-affinity receptor (FcεRI) [5–7] using monoclonal antibodies has been shown to be promising for the therapy of allergy [8,9]. Inhibition studies using ε-chain peptides [10,11] and the determination of the crystal structure of the IgE–FcεRI complex [12] mapped the two binding sites of FcεRI to the N-terminal region of the Cε3 domains of both IgE heavy chains. Consistently with this requirement, omalizumab as well as other non-anaphylactogenic anti-IgE antibodies have been shown to recognize epitopes overlapping with the binding site of FcεRI [13]. Indeed, binding of these epitopes is thought to be of key importance for any bivalent IgE inhibitor, as the binding of anti-IgE antibodies to other epitopes allows the cross-link of IgE molecules already bound by its receptors, leading to the activation and degranulation of the underlying effector cells [14].

Murine anti-IgE antibodies of such epitope specificity are capable to neutralize IgE *in vitro* [7,13,15]. One such antibody binding serum-free IgE is omalizumab (Xolair®, Novartis Pharmaceuticals, Germany; Genentech Inc., USA). It has been humanized [16] and was shown to efficiently decrease serum IgE levels *in vivo* by 85–95%. The density of FcεRI on basophils was simultaneously declined [17]. Treatment led to lower exacerbations and reduced the need for systemic corticosteroid therapy in clinical trials [8,18–22]. However, as only 1000–1500 receptors need to be cross-linked to initiate an allergic reaction [17,23], high doses (ranging from 75 mg to 375 mg every two or four weeks, depending on the patient's body weight and total serum IgE levels) of omalizumab are required to achieve successful IgE neutralization, rendering this therapy useful and cost-effective only for a restricted group of patients with severe asthma [24]. Besides optimized cost-effectiveness, increased potency would not only allow the existing anti-IgE patients to be treated in a less-frequent and lower-dosing regimen, but also broaden the group of patients to those suffering from asthma but currently not matching the inclusion criteria for omalizumab treatment.

Numerous attempts have been made to create molecules with higher potency, either by modifying the immunoglobulin structure directly [25] or by developing alternative binding scaffolds based on non-immunoglobulin molecules [26]. DARPins (designed ankyrin repeat proteins) represent a promising approach of such novel binding scaffolds [26,27]. DARPins are designed proteins derived from natural ankyrin repeat proteins. They are composed of several repeat subunits each representing a binding domain. The assembled protein consists of two capping repeats intervened by two or more binding modules that are randomly modified on their surface. Combinatorial libraries with DARPins of varying repeat numbers have been constructed and have been used for selections against a wide range of targets [28–34], reviewed recently [35,36]. In the field of allergy, we have recently characterized bispecific DARPins that

* Corresponding author. Tel.: +41 31 632 03 34; fax: +41 31 381 57 35.
E-mail address: monique.vogel@iib.unibe.ch (M. Vogel).

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efficiently antagonized the IgE receptor [28]. The isolated DARPins exhibited high specificity, affinity and selectivity and were shown to bind their target via the designed randomized residues [37].

In this study we applied DARPin libraries for the selection of binders to the constant region of IgE *in vitro* using ribosome display [38]. Specificity and affinity of the isolated DARPins were verified in enzyme-linked immunosorbent assays (ELISAs) and surface plasmon resonance (SPR) experiments, respectively. We produced bivalent and bispecific DARPins by cloning two DARPins joined by a glycine–serine linker. These constructs showed higher potency in inhibiting proinflammatory mediator release using *in vitro* functional assays compared to monoclonal anti-IgE antibodies. Further studies can now be performed to explore the therapeutic potential of DARPins in atopic diseases.

2. Materials and methods

2.1. IgE, anti-IgE and IgE receptor preparations

IgE-Sus11 is a monoclonal IgE from a hybridoma cell line [39,40]. The myelomas IgE-Zavazal and IgE-PS are kind gifts from Dr. V. Zavázal (Pilsen, Czech Republic) [41] and Drs. T. Ishizaka and K. Ishizaka (La Jolla, CA, USA), respectively. IgE-JW8 is a chimeric immunoglobulin, consisting of a human IgE constant and a mouse variable region, as previously described [42].

The following anti-human IgE antibodies were used: the murine monoclonal IgG BSW17 [7], the humanized monoclonal IgG omalizumab [16] (Xolair®, Novartis Pharmaceuticals, Germany; Genentech Inc., USA), and the murine monoclonal IgG Le27 [43].

Two different recombinant FcεRIα constructs were used: first, a fusion protein of human serum albumin flanked twice by the extracellular part of the alpha chain of the high-affinity IgE receptor (bivalent, bFcεRIα), a kind gift of Dr. H. Kocher (Novartis Pharma, Basel, Switzerland) [44], and second, a fusion protein of the extracellular part of FcεRIα to a human IgG_1 Fc (monovalent, mFcεRIα), a kind gift from Dr. M. Fux (Glenmark Pharmaceuticals S.A., La Chaux-de-Fonds, Switzerland).

2.2. DARPin libraries

Libraries containing either two or three randomized internal repeats (designated as N2C or N3C, respectively) flanked by constant N- and C-terminal capping repeats were obtained from Molecular Partners AG (Zürich-Schlieren, Switzerland). The generation of these DARPin libraries has been described elsewhere [27].

2.3. In vitro selection by ribosome display

The PCR-amplified N2C and N3C DARPin libraries were transcribed and translated *in vitro* and subjected to four standard rounds of ribosome display selection as described earlier [26,28,38,45]. To avoid the selection of anti-idiotypic and anti-allotypic DARPins, subsequent selection rounds were performed with different IgE hybridomas and myelomas. After each round, the mRNA of the selected libraries was processed as described [28,33].

In the fifth ribosome display round, the libraries were subjected to off-rate selection, thereby increasing selection pressure. For this purpose, IgE-Sus11 was biotinylated using the EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce, MA, USA) according to the manufacturer's manual. 1 nM biotinylated IgE-Sus11 was incubated for 1 h with the translated libraries before the addition of a 500-fold molar excess of non-biotinylated IgE-Sus11 for a further hour. The libraries were then transferred to microtiter plates pre-coated with 0.5 μg neutravidin and incubated for 1 h at 4 °C in order to capture the biotinylated binders complexed with IgE. After this last selection round, the libraries were processed as described above.

2.4. Expression and analysis of selected binders

From each library, single clone *Escherichia coli* crude extracts were generated as described previously by our group [28], and their IgE specificity was analyzed by ELISA (data not shown). Based on this assay, four DARPins were selected for further characterization.

2.5. Sequencing, protein production and purification of monovalent, bivalent and bispecific DARPins

DNA sequencing, expression in *E. coli* and immobilized metal affinity chromatography (IMAC) of the selected DARPins was performed as previously described [28]. For β-hexosaminidase release assay, the proteins were further purified by preparative size exclusion chromatography, on a HiLoad 26/60 Superdex-75 prep grade (Amersham Pharmacia Biotech, USA) column as described [27]. Cloning and production of bivalent and bispecific DARPins was carried out as previously published by our group [28].

2.6. Isotype specificity analysis of selected DARPins

To assess isotype specificity, the selected monovalent DARPins were tested on different immunoglobulin isotypes. IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, IgM (The Binding Site, product numbers BP078, BP080, BP082 and BP084, BP086 and BP091, respectively) and three different IgEs (IgE-Sus11, IgE-PS and IgE-Zavazal) were immobilized at 5 μg/ml in 100 μl PBS. Bound DARPins were detected with a biotinylated anti-polyhistidine antibody (R&D Systems, Minneapolis, USA) followed by a streptavidin–horseradish peroxidase (HRP) conjugate (DakoCytomation, Glostrup, Denmark).

2.7. Reactivity of DARPins to IgE in human serum

To investigate the binding efficacy of DARPins *in vivo* we tested whether they can recognize IgE in human serum. The bivalent anti-IgE DARPin (E2_79/E2_79) and the controls (an unrelated non-specific bivalent DARPin D2_7/D2_7, and the murine anti-human IgE specific antibody Le27) were coated at 5 μg/ml in 100 μl PBS. IgE-Sus11 was added at increasing concentrations diluted either in PBS/casein or in human serum. Bound IgE was detected using HRP-conjugated goat anti-human IgE (The Binding Site). TMB (3,3′,5,5′-tetramethylbenzidine) was used as substrate to develop the ELISA. The reaction was stopped after 5 min with 1 M sulfuric acid and the absorbance was measured at OD 450 nm in a standard ELISA reader (BIO-TEK EL808, BioTek, Bad Friedrichshall, Germany).

2.8. Determination of kinetic parameters by SPR

SPR measurements were performed on a Biacore X instrument (Biacore AB, Uppsala, Sweden) using HBS-EP (10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20) as running buffer. IgE-Sus11 immobilized on one flow cell of a CM5 sensor surface chip to a final density of 2500 resonance units (RU) according to the manufacturer's protocol (amine coupling). The second flow cell was used as control for unspecific binding to the CM5 sensor surface. All measurements were done at a flow rate of 10 μl/min at room temperature. The DARPins E2_79, E3_54, E2_79/E2_79 and E2_79/E3_54 were injected for 3 min at concentrations ranging from 100 nM to 3.125 nM, including a HBS-EP buffer control. The off-rate was measured for additional 3 min. Regeneration of the sensor chip surface was performed by injection of 5 μl 25 mM NaOH. Kinetic data evaluation was performed using global fitting and the Lang-