

Intranasal immunisation using recombinant *Lactobacillus johnsonii* as a new strategy to prevent allergic disease

Lorenz Scheppler^a, Monique Vogel^{a,*}, Pamela Marti^a, Lorenz Müller^a,
Sylvia M. Miescher^{a,b}, Beda M. Stadler^a

^a Institute of Immunology, Inselspital, 3010 Bern, Switzerland

^b ZLB Behring, Wankdorfstrasse 10, 3000 Bern 22, Switzerland

Received 21 April 2004; received in revised form 13 August 2004; accepted 16 August 2004

Available online 19 September 2004

Abstract

We have previously demonstrated the induction of a specific anti-IgE response in vivo by parenteral immunisation of rhesus monkeys using short IgE mimotopes or an anti-idiotypic antibody mimicking an IgE epitope. Such specific anti-IgE responses may be of clinical benefit for atopic patients. In this study, we examined the potential for a more convenient therapy via mucosal immunisation using live recombinant *Lactobacillus johnsonii* (Lj) as a vaccine delivery vehicle. Either an anti-idiotypic scFv or an IgE mimotope were expressed on the surface of Lj as fusion proteins using the cell wall anchored proteinase PrtB from *Lactobacillus delbrueckii* subsp. *bulgaricus*. The recombinant Lj were shown to express the heterologous fusion proteins and were specifically recognised by the corresponding anti-human IgE monoclonal antibody. Subcutaneous and intranasal immunisation of mice with recombinant Lj, expressing these fusion proteins induced a systemic IgG response against human IgE. Our data suggest that recombinant Lactobacilli expressing IgE epitopes may represent a novel means of vaccination to induce a beneficial anti-IgE response.

© 2004 Elsevier Ltd. All rights reserved.

Keywords: *Lactobacillus johnsonii*; IgE mimotope; Anti-idiotypic single chain fragment variable

1. Introduction

The incidence of atopic disease has increased considerably in most Western countries during the last 30 years. New therapies are required independent of the initiating allergen which would have a broader application to many different patient groups. A promising approach has been the development of non-anaphylactogenic anti-IgE antibodies aimed at targeting IgE which plays a key role in initiating the symptoms of atopic disease. These anti-IgE antibodies recognize epitopes close to the binding site of IgE on the high affinity IgE receptor (FcεRI) [1,2]. One of these anti-IgE antibodies, omalizumab is currently used to treat allergic patients by passive immunisation [1–8]. However, passive immunisation requires continuous treatments with relatively high amounts

of monoclonal antibodies resulting in high costs. Therefore, an active immunisation strategy aimed at neutralising IgE was envisaged by using IgE epitopes to induce a natural autoimmune response, resulting in the formation of autoantibodies that may have the same effect as passively administered non-anaphylactogenic anti-IgE antibodies. In order to isolate therapeutically applicable, immunogenic IgE epitopes a non-anaphylactogenic monoclonal anti-IgE antibody BSW17 was used as a screening tool [9,10] on random peptide phage display libraries [11,12]. Two peptides (Cε3mim and Cε4mim) were isolated which mimicked part of the IgE epitope recognised by BSW17 and were therefore called IgE mimotopes. In addition, an anti-idiotypic Fab (Fab α-Id-B43) was isolated [13] which mimicked the same region as the IgE mimotopes but in addition covered a larger area on IgE. Both the mimotopes [14] and the anti-idiotypic Fab were used for parenteral immunisation of rhesus monkeys and were shown in a passive cutaneous anaphylaxis (PCA) test to completely

* Corresponding author. Tel.: +41 31 632 03 34; fax: +41 31 381 57 35.
E-mail address: monique.vogel@dkf6.unibe.ch (M. Vogel).

abolish PCA reactivity after allergen challenge (manuscript in preparation).

In this study, we analysed the ability of *Lactobacillus johnsonii* (Lj) to express Cε4mim and α-IdscFv2, an anti-idiotypic single chain Fragment variable (scFv) derived from Fab α-Id-B43 to induce an anti-IgE response after active, mucosal immunisation. *Lactobacillus* strains are attractive candidates as carriers for mucosal vaccination. Since centuries, they have been used for food processing and food preservation and are therefore considered to be safe organisms for human consumption. Several systems have been described using *Lactobacillus* as a carrier for expressing foreign antigens and for mucosal immunisation. Zegers et al. [15] have reported the use of *Lactobacillus casei* to express the protective antigen of *Bacillus anthracis* as an oral prototype vaccine against anthrax. In addition, the use of tetanus toxin fragment C (TTFC) as a model antigen for expression on *Lactobacillus plantarum* [16,17] or on *L. casei* [18] and the induction of an anti-TTFC specific immune response after mucosal immunisation has been described. We have reported the use of Lj to express mimotopes fused to the cell wall anchored proteinase PrtB [19] and the induction of a local and a systemic immune response against these heterologous proteins after oral immunisation [20].

In the present study, we show that both an anti-idiotypic scFv and an IgE mimotope could be expressed as a fusion protein with PrtB on the surface of Lj and induced anti-IgE antibodies after subcutaneous and intranasal administration. In contrast to other reports, we demonstrate here that Gram-positive bacteria expressing anti-idiotypic single chain Fragment variable (scFv) may be used to act as immunomodulators. A mucosal anti-IgE vaccine based on recombinant Lj would not only be much cheaper than the currently used passive administration of monoclonal antibodies, but would also avoid disadvantages associated with parenterally administered vaccines.

2. Materials and methods

2.1. Plasmids and oligonucleotides

Plasmid pComb3His was reconstructed based on pComb3H [12] by inserting 6His tags at the *SpeI/NheI* sites. Plasmid pComb3-α-Id-B43 contains the sequence coding for Fab α-Id-B43. Plasmid pMD112 was constructed by inserting into pNZ124 [21] the cell wall anchored proteinase (prtB) gene [19] of *Lactobacillus delbrueckii* subsp. *bulgar-*

icus and was isolated from transformed *Lactococcus lactis* as described recently [20]. The linker sequences used for the construction of the anti-idiotypic scFv fragments were designed as described elsewhere [22–25] (Table 1). All oligonucleotides were synthesised by Microsynth GmbH (Balgach, Switzerland).

2.2. Bacteria and reagents

Bacterial strain XL-1 Blue was purchased from Stratagene (San Diego, USA). *L. johnsonii* strain NCC2754 (Lj) were grown in MRS broth (Difco Laboratories, Detroit, USA) containing 1% Glucose. Chloramphenicol (Fluka Chemie AG, Buchs, Switzerland) was used at a concentration of 10 µg/ml. All restriction enzymes were purchased from Roche Diagnostics GmbH (Rotkreuz, Switzerland).

2.3. Antibodies and antigens

Affinity-purified rabbit anti-PrtB antibodies were purchased from Eurogentech (Seraing, Belgium). Rabbit anti-human Cε4mim [10] and anti-human IgE antibody BSW17 [9,26] were prepared in house as previously described. Rabbit anti-α-Id-B43 was prepared in our laboratory by immunising rabbits using Fab α-Id-B43 [13]. Horseradish peroxidase (HRP) conjugated sheep anti-mouse IgG was purchased from Cappel (ICN, Switzerland). HRP-conjugated goat anti-rabbit antibodies were obtained from Nordic Immunology Laboratories (Tilberg, The Netherlands). Human myeloma IgE Savasal was a kind gift from Dr. V. Savasal (Pilsen, Czech Republic) and was purified on a MonoQ ion exchange column using an FPLC system (Pharmacia Biotech AG, Dübendorf, Switzerland) and used at a concentration of 10 µg/ml.

2.4. Production of anti-idiotypic scFv fragments in *Escherichia coli* XL-1 Blue

V_H and V_L sequences of Fab α-Id-B43 [13] and the linker sequences used for the construction of the anti-idiotypic scFv fragments were published elsewhere [22–25] and are shown in Table 1. The V_H and V_L sequences of the anti-idiotypic scFv fragments were amplified separately from plasmid pComb3-α-Id-B43 using primer pairs specific for the N- and C-terminal of either the V_H or the V_L regions. To assemble V_H and V_L fragments, overlapping oligonucleotides encoding different linkers (Table 1) were inserted between heavy and light chain in a PCR reaction. For subsequent cloning, the assembled products were PCR amplified

Table 1
Linkers for the construction of anti-idiotypic scFv fragments

ScFv designation ^a	Linker sequence	Linker description	Reference for linkers
α-IdscFv1	GGGGSGGGSGGGGS	Flexible linker	[22,23]
α-IdscFv2	PNGASNSGSAPDTSSAPGSQ	β-Turn linker	[24]
α-IdscFv3	YPRSIYIRRRHPSPSLTT	Random linker	[25]

^a Sequences of V_L and V_H of Fab α-Id-B43 are already published [13].

with flanking primers containing *SacI* and *SpeI* restriction sites.

The resulting DNA fragments encoding either α -IdscFv1, α -IdscFv2 or α -IdscFv3 were then digested with *SacI* and *SpeI* restriction enzymes and inserted into plasmid pComb3His digested with *SacI* and *SpeI*. Ligated plasmid DNA was used to transform *Escherichia coli* XL-1 Blue. Positive clones were tested for the presence of the corresponding α -IdscFv DNA by PCR and sequencing analysis (data not shown) and were subjected to large-scale production. Transformed XL-1 Blue were cultivated in 1000 ml of super broth (SB) at pH 7.0 in the presence of ampicillin (50 μ g/ml) at 37 °C until an OD at 600 nm of 1.0 was reached. Subsequently, protein synthesis was induced by 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside; Promega, Madison, WI). The bacteria were further cultured by shaking (220 rpm) for 4 h at 37 °C. The culture supernatant was collected and purified on a BD TALON™ Metal Affinity Resin (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

2.5. Screening of anti-idiotypic scFv fragments by anti-human IgE antibodies

The anti-idiotypic scFv fragments were tested by ELISA using Costar EIA/RIA half-well plates (Costar, Cambridge, MA) coated for 4 h at 37 °C with α -IdscFv1, α -IdscFv2 or α -IdscFv3 at a concentration of 10 μ g/ml. IgE Savasal coated at the same concentration was used as a control. Wells were blocked for 2 h at 37 °C with PBS/5% BSA and incubated with either BSW17 (50 μ g/ml), rabbit anti-human C ϵ 4mim (10 μ g/ml) or rabbit anti- α -Id-B43 (1:1000) for 4 h at 37 °C. Bound antibodies were detected by using HRP-conjugated sheep anti-mouse IgG (Fc) or HRP-conjugated goat anti-rabbit IgG (Fc) antibodies incubated for 1.5 h at 37 °C. Tetramethylbenzidine (3,3',5,5'-tetramethylbenzidine, Fluka, Buchs, Switzerland) was used as substrate. The absorbance was read after 5 min at 450 nm on a Molecular Devices ELISA reader (Paul Bucher, Basel, Switzerland).

2.6. Generation of Lj transformants expressing PrtB fusion proteins

Recombinant Lj expressing either a tetanus mimotope (LjTT) or a C ϵ 4mim (Lj ϵ 4), recognised by the monoclonal mouse anti-human IgE antibody BSW17 [9] have been described elsewhere [20]. Recombinant Lj expressing an α -IdscFv2-PrtB fusion protein (Lj α -IdscFv2) was produced by first subjecting DNA encoding α -IdscFv2 to a PCR reaction using primers containing *PvuI* and *NheI* restriction sites. Plasmid pMD112 was digested with *PvuI* and *NheI*, gel purified and ligated to the PCR product encoding α -IdscFv2. Ligated DNA was used to transform electrocompetent Lj and transformants were plated on MRS plates containing chloramphenicol (10 μ g/ml). Clones were tested for the presence

of α -IdscFv2 DNA by PCR and sequencing analysis (data not shown).

2.7. Gel electrophoresis and Western immunoblots

Transformed Lj α -IdscFv2 and LjTT bacteria were grown overnight in 25 ml medium containing 10 μ g/ml chloramphenicol. Bacterial cells were harvested by centrifugation at 3000 $\times g$ for 15 min at 4 °C and washed with 5 ml TBS. After recentrifugation, the bacterial pellet was resuspended in 450 μ l TBS and 150 μ l 4 $\times$ non-reducing sample buffer (80 mM Tris-HCl, pH 6.8, 2.5% SDS, 0.15% glycerol, 0.005% bromophenol blue). Aliquots (20 μ l) were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (6% acrylamide, 0.5 M Tris-HCl, pH 8.8) and run in a 25 mM Tris, 192 mM glycine buffer (pH 8.3) at 100 V for 60 min. The gels were stained with Bio-Safe Coomassie Stain (Bio-Rad Laboratories, Reinach, Switzerland) or transferred electrophoretically onto Protran BA 83 nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). After transfer, the membranes were blocked with PBS/5% BSA for 2 h at RT and incubated with affinity purified rabbit anti-PrtB antibodies (1:2000) overnight at RT followed by incubation for 3 h at RT with a 1:1000 dilution of HRP-conjugated goat anti-rabbit IgG. Immunoblots were developed with 4-chloro-1-naphtol for 2 min.

2.8. Catching ELISA using recombinant Lj

Transformed bacteria (Lj α -IdscFv2, Lj ϵ 4 and LjTT) and wild type Lj were grown overnight in 50 ml medium containing 10 μ g/ml chloramphenicol. Bacterial cells were harvested by centrifugation at 3000 $\times g$ for 15 min at 4 °C, washed with 5 ml TBS and recentrifuged. Finally, the bacterial pellet was resuspended in 1 ml TBS/10% (v/v) PBS/2% FCS/0.05% Tween 20. Fifty microliters of this bacterial suspension (approximately 5 $\times 10^7$ cells) were overlain on Costar EIA/RIA half-well plates (Costar, Cambridge, MA) coated with 50 μ g/ml anti-human IgE BSW17 overnight at 4 °C. Unbound bacteria were washed off the wells using PBS/0.1% Tween 20. Wells were then incubated with rabbit anti-PrtB antibodies diluted 1:2000 in PBS/2% FCS/0.05% Tween 20 for 4 h at 37 °C. Bound antibodies were detected using HRP-conjugated goat anti-rabbit IgG (Fc) antibodies. Tetramethylbenzidine (Fluka) was used as substrate. The absorbance was read after 5 min at 450 nm on a Molecular Devices ELISA reader (Paul Bucher).

2.9. Immunisation of mice

Groups of five female BALB/c mice (8 weeks old) were immunised subcutaneously with 10⁸ live LjTT, Lj ϵ 4 or Lj α -IdscFv2 at days 0, 21 and 43. The bacterial suspension used for the immunisation was mixed 1:2 with Al(OH)₃. For intranasal immunization, three weekly doses of 10⁹ live LjTT, Lj ϵ 4 or Lj α -IdscFv2 were administered at days 0, 1 and 2 to

three groups, each of five non-anaesthetized mice in a volume of 12 μ l/mouse. Booster immunisations were given at days 21, 22, 23 and 43, 44, 45. Sera were collected by retro-orbital bleeding at days 52 (subcutaneous immunisations) and 56 (intranasal immunisations), respectively [16].

2.10. Evaluation of antibody responses

The antibody response was measured by ELISA. Costar EIA/RIA half-well plates (Costar, Cambridge, MA) were coated overnight at 37 °C with either 10 μ g/ml human IgE Savasal or 10 μ g/ml soluble α -IdscFv2. Sera were tested at dilutions of 1:50, 1:100 and 1:200 by incubation on the coated wells at 37 °C for 4 h. Bound antibodies were detected using HRP-conjugated sheep anti-mouse IgG (Fc) incubated for 1.5 h at 37 °C. Tetramethylbenzidine (Fluka) was used as substrate. The absorbance was read after 6 min at 450 nm on a Molecular Devices ELISA reader (Paul Bucher). Serum of non-immunised mice were used as negative controls (background) and subtracted from serum signals. Absorbance in these samples was never higher than OD 0.1.

3. Results

3.1. Production of soluble anti-idiotypic scFv fragments in *E. coli* XL-1 Blue

We have previously isolated an anti-idiotypic Fab (Fab α -Id-B43), mimicking part of the third and fourth constant domains of human IgE [13]. In order to develop a mucosal anti-IgE vaccine, we expressed Fab α -Id-B43 on the surface of Lj. The binding site of Fab α -Id-B43 was constructed as a single chain fragment variable (α -IdscFv), because Gram-positive bacteria lack an oxidative site, where heavy and light chain of an Fab could assemble.

As the linker used for the construction of a scFv influences its correct folding [24,25], three anti-idiotypic scFv fragments were tested using different linkers. Thus, annealed overlapping oligonucleotides encoding the different linkers for α -IdscFv1 ((Gly₄-Ser)₃-linker [22,23]), α -IdscFv2 (β -turn-promoting linker [24]), or α -IdscFv3 (random linker [25]) were used for assembly of V_H and V_L of α -Id-B43 in a PCR reaction (Table 1). In a subsequent PCR reaction, restriction sites were added and the final PCR products encoding the three α -IdscFv1, 2 and 3 were inserted into the *Sac*I and *Spe*I restriction sites of pComb3His, which contains 6His tags. The recombinant plasmids were transformed into electrocompetent *E. coli* XL-1 Blue. Soluble anti-idiotypic scFv fragments were produced and isolated on a nickel affinity column.

3.2. Screening for correctly folded anti-idiotypic scFv fragments

Analysis of the isolated anti-idiotypic scFv fragments was performed using α -IdscFv1, α -IdscFv2 and α -IdscFv3 as

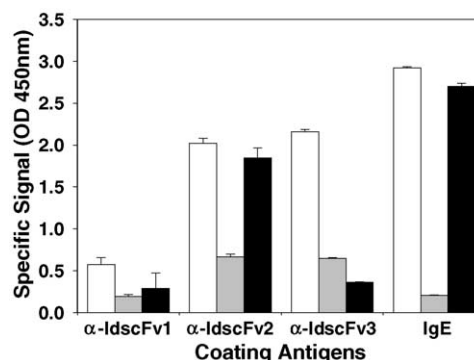


Fig. 1. Detection of the anti-idiotypic scFv fragments by anti-human IgE antibodies. Wells were coated with α -IdscFv1, α -IdscFv2, α -IdscFv3 or with human IgE Savasal at a concentration of 10 μ g/ml. Wells were subsequently incubated with either 50 μ g/ml of monoclonal mouse anti-human IgE (BSW17, white bars), with a rabbit anti-human Cε4mim serum (diluted 1:1000, grey bars) or a rabbit anti- α -Id-B43 serum (BSW17-like, diluted 1:1000, black bars). Bound antibodies were detected by using either HRP-conjugated sheep anti-mouse IgG (Fc) or HRP-conjugated goat anti-rabbit antibodies. Bars represent mean values ($\pm$ S.D.) from three individual experiments.

coating antigens in an ELISA. Human IgE was used as a control and was analysed on the same ELISA plate. Fig. 1 shows the recognition of the anti-idiotypic scFv fragments by different antibodies reacting with human IgE. α -IdscFv2, α -IdscFv3 and the control IgE were well recognised by the monoclonal anti-human IgE BSW17 and slightly recognised by the rabbit anti-human Cε4mim serum, whereas the recognition of α -IdscFv1 was only minimal. In contrast, the rabbit anti- α -Id-B43 serum (BSW17-like) only recognised α -IdscFv2 and the control IgE, but not α -IdscFv1 and α -IdscFv3. As the control IgE showed the same pattern of recognition as α -IdscFv2, only α -IdscFv2 was used in the further experiments.

3.3. Transformation of lactic acid bacteria

For development of an oral vaccine using *L. johnsonii* (Lj), the plasmid pMD112 was chosen as the expression vector. This plasmid contains the entire structural gene of the cell wall anchored proteinase (PrtB) from *L. delbrueckii* subsp. *bulgaricus*. DNA encoding α -IdscFv2 was amplified in a PCR using primers containing *Pvu*I and *Nhe*I restriction sites. The final PCR product coding for α -IdscFv2 was inserted at the *Nhe*I and *Pvu*I restriction sites which are located in the active site of PrtB. In addition, an ε4 mimotope (Cε4 mim), an IgE decapeptide [10] and for control purposes a tetanus toxin mimotope (TTmim), a peptide of 11 amino acids derived from tetanus toxin were all cloned in the same way as α -IdscFv2, as described recently [20]. The resulting plasmids pMD112 α -IdscFv2, pMD112ε4 and pMD112TT thus code for proteins inserted into the sequence of PrtB that are expressed as fusion proteins on the bacterial cell surface.

Lj was transformed with the recombinant plasmids pMD112 α -IdscFv2, pMD112ε4 and pMD112TT (Table 2).

Table 2
Bacterial clones and designations

Clone designation	Bacterial strain	Plasmid used for transformation	Peptide fused to PrtB	Expressed fusion protein ^a
Lj	<i>Lactobacillus johnsonii</i>	None	None	None
LjTT	<i>Lactobacillus johnsonii</i>	pMD112TT	TTmim	TTmim-PrtB
Ljε4	<i>Lactobacillus johnsonii</i>	pMD112ε4	Cε4mim	Cε4mim-PrtB
Ljα-IdscFv2	<i>Lactobacillus johnsonii</i>	pMD112α-IdscFv2	α-IdscFv2	α-IdscFv2-PrtB

^a Expression was monitored by Western blot and ELISA (see Section 3, Fig. 2 and [20]).

DNA was prepared from chloramphenicol resistant transformants and tested by PCR and sequencing analysis for the presence of the recombinant plasmids using primers specific for the different peptides and the wild type proteinase PrtB (data not shown).

3.4. Expression of the recombinant PrtB fusion proteins in Lj

The expression of the α-IdscFv2-PrtB fusion protein in Lj was analysed by SDS-PAGE Western blot and ELISA. Both Ljε4 and LjTT were already shown to express recombinant PrtB fusion proteins [20] and were used as controls in addition to wild type Lj. Fig. 2A demonstrates that α-IdscFv-PrtB fusion protein was substantially less expressed than TTmim-PrtB. This in contrast to ε4mim-PrtB which has been shown in a previous study to be expressed in a comparable amount as TTmim-PrtB. However, breakdown products with molecular weights between 90 and 210 kDa were observed in either recombinant LjTT or Ljα-IdscFv2 transformants. On the Western blot, these bands showed reactivity with rabbit-anti PrtB serum (Fig. 2B), indicating that the PrtB protein was expressed by the Lj bacteria and that these bands correspond to full length (MW 190 kDa for LjTT and 212 kDa for Ljα-IdscFv2) and to fragments of PrtB. As α-IdscFv2 is a conformational epitope, the bacterial clones were tested for correctly folded α-IdscFv2-PrtB fusion protein in a catching ELISA. BSW17 was coated onto ELISA plates and was overlaid with either Lj, LjTT, Ljε4 or Ljα-IdscFv2. Unbound bacteria were washed off the wells, and bound bacteria were detected using rabbit anti-PrtB antibodies. As shown in Fig. 2C (third and fourth bar), only Ljε4 and Ljα-IdscFv2 bound to BSW17 coated on the solid phase, indicating that α-IdscFv2 was expressed as a fusion protein with PrtB. Only a marginal reactivity was observed with either wild type Lj or recombinant LjTT.

3.5. Subcutaneous immunisation of mice with Ljα-IdscFv2

Sera of mice immunised subcutaneously with wild type and recombinant *L. johnsonii* bacteria were tested by ELISA 52 days after the first immunisation using soluble α-IdscFv2 (Fig. 3A) or human myeloma IgE ("Savazal") on the solid phase (Fig. 3B). Fig. 3A shows that different dilutions of sera of mice immunised with Ljα-IdscFv2 recognise soluble α-IdscFv2. Weaker reactivities to soluble α-IdscFv2 were

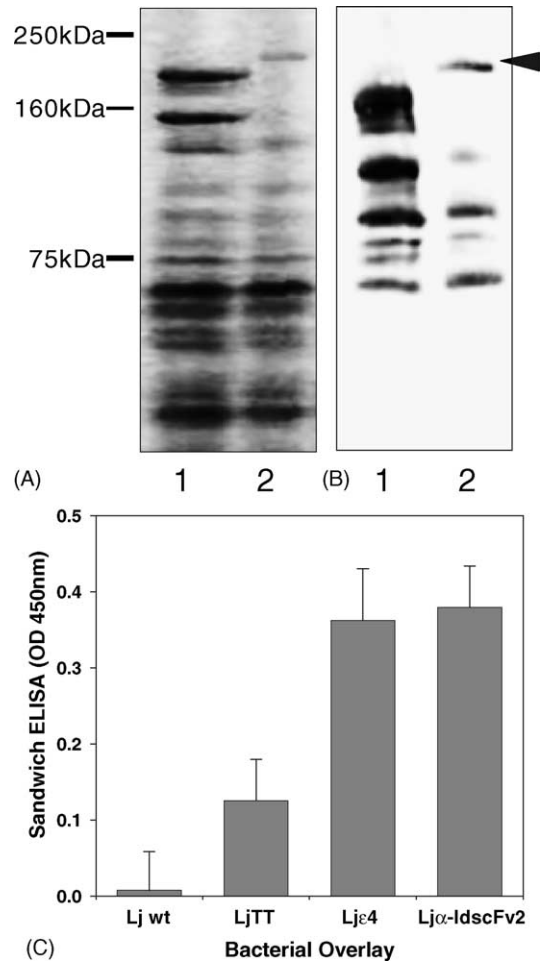


Fig. 2. Recombinant expression of α-IdscFv2-PrtB fusion protein in Lj. (A and B) Whole bacterial cell lysates from overnight cultures of LjTT (lane 1) or Ljα-IdscFv2 (lane 2) were separated by SDS-PAGE 6% and stained with Bio-Safe Coomassie Stain (A) or transferred to nitrocellulose filters and incubated with affinity purified rabbit anti-PrtB antibodies (B). Bound antibodies were detected with HRP-conjugated goat anti-rabbit IgG (Fc) antibodies. The arrow indicates the height of full-length α-IdscFv2-PrtB. (C) Wells were coated with BSW17 (anti-human IgE) at a concentration of 50 μg/ml and overlaid with 5×10^7 wild type Lj (Lj wt), or recombinant Lj bearing either a tetanus-derived mimotope (LjTT), an IgE (Cε4) derived mimotope (Ljε4) or α-IdscFv2 fused to PrtB (Ljα-IdscFv2). Unbound bacteria were washed off and wells were incubated with affinity purified rabbit anti-PrtB antibodies. Bound antibodies were detected with HRP-conjugated goat anti-rabbit IgG (Fc) antibodies. Bars represent mean values ($\pm$ S.D.) from three individual experiments.

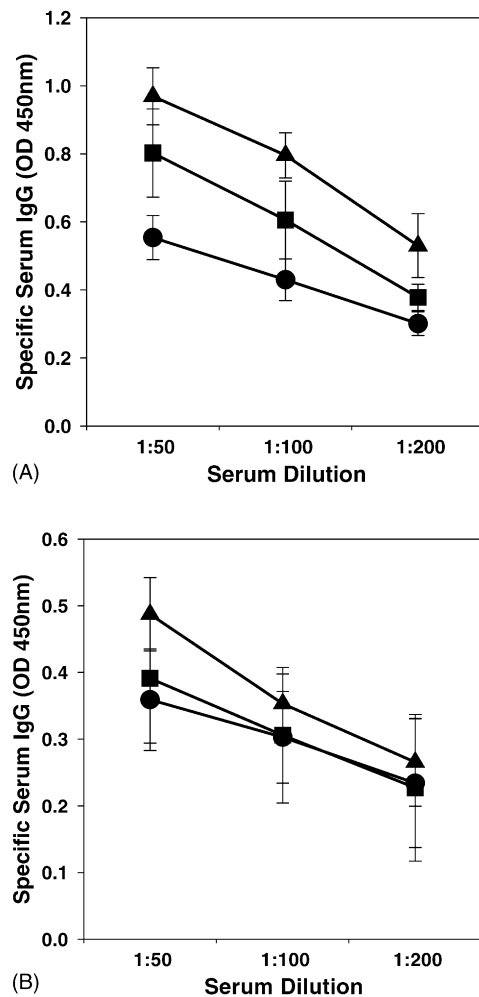


Fig. 3. Binding specificities of serum IgG from mice subcutaneously immunised with Ljα-IdscFv2, Ljε4, or LjTT. Wells were coated with either 10 µg/ml soluble α-IdscFv2 produced in *Escherichia coli* XL-1 Blue (A) or with human IgE Savasal at a concentration of 10 µg/ml (B). Wells were incubated with different dilutions of sera collected from mice immunised subcutaneously with either Ljα-IdscFv2 (triangles), Ljε4 (squares) or LjTT (circles). Bound antibodies were detected using HRP-conjugated sheep-anti-mouse IgG (Fc) antibodies. Values represent mean values (±S.D.) for five mice. In (A) values obtained with the three dilutions (1:50, 1:100, 1:200) of sera of mice immunized with either Ljα-IdscFv2 ($p < 0.01$) or Ljε4 ($p < 0.01$) are significantly higher than those obtained with LjTT. In (B) only the value obtained with one dilution (1:50) of sera of mice immunised with Ljα-IdscFv2 ($p < 0.01$) is significantly higher than that obtained with LjTT.

also observed with sera of mice immunised either with Ljε4 or with the control strain LjTT. According to the Student's *t*-test the reactivities observed with sera of mice immunised with either Ljα-IdscFv2 or Ljε4 were significantly different from the ones observed with sera of mice immunised with LjTT. These results indicate that a specific response was induced against α-IdscFv2 after immunisation with either Ljα-IdscFv2 or Ljε4. As shown in Fig. 3B only sera of mice immunised with Ljα-IdscFv2 react with human IgE. The reactivity observed with the sera of mice immunised with Ljε4 was not statistically different from that obtained using sera of mice immunised with LjTT. Thus, a specific reaction against

IgE was induced only upon immunisation of mice with Ljα-IdscFv2.

3.6. Intranasal immunisation of mice with Ljα-IdscFv2

Sera of mice immunised intranasally with wild type and recombinant *L. johnsonii* bacteria were tested by ELISA 52 days after the first immunisation using soluble α-IdscFv2

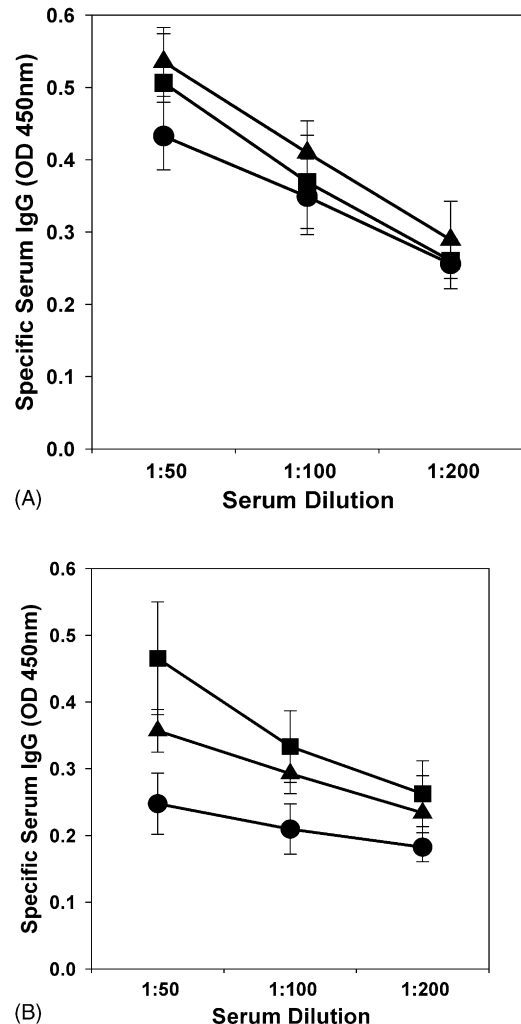


Fig. 4. Binding specificities of serum IgG from mice intranasally immunised with Ljα-IdscFv2, Ljε4, or LjTT. Wells were coated with either 10 µg/ml soluble α-IdscFv2 produced in *Escherichia coli* XL-1 Blue (A) or with human IgE Savasal at a concentration of 10 µg/ml (B). Wells were incubated with different dilutions of sera collected from mice immunised intranasally with either Ljα-IdscFv2 (triangles), Ljε4 (squares) or LjTT (circles). Bound antibodies were detected using HRP-conjugated sheep-anti-mouse IgG (Fc) antibodies. Values represent mean values (±S.D.) for five mice. In (A) values obtained with two dilutions (1:50 and 1:100) of sera of mice immunized with Ljα-IdscFv2 ($p < 0.01$ for 1:50 and $p < 0.05$ for 1:100) and with one dilution (1:50) of sera of mice immunized with Ljε4 ($p < 0.05$) are significantly higher than those obtained with LjTT. In (B) values obtained with the three dilutions (1:50, 1:100 and 1:200) of sera immunized with either Ljα-IdscFv2 ($p < 0.01$ for 1:50 and 1:100 and $p < 0.05$ for 1:200) or Ljε4 ($p < 0.01$ for dilutions 1:50, 1:100 and 1:200) are significantly higher than those obtained with LjTT.

(Fig. 4A) or human IgE Savazal on the solid phase (Fig. 4B). As shown in Fig. 4A soluble α -IdscFv2 reacts with sera of mice immunised intranasally with Lj α -IdscFv2 but also to a lesser extent to sera of mice immunised with either Lj ϵ 4 or the control strain LjTT. Based on the Student's *t*-test, only sera of mice immunised with either Lj α -IdscFv2 or Lj ϵ 4 showed a specific reaction to soluble α -IdscFv2. Lj α -IdscFv2 induced a stronger specific antibody response to α -IdscFv2 than Lj ϵ 4 as shown by serial dilution of the serum. In contrast, Fig. 4B shows that human IgE was better recognised by sera of mice immunised with Lj ϵ 4 than with Lj α -IdscFv2 indicating that intranasally Lj ϵ 4 induced a stronger anti-IgE response than Lj α -IdscFv2. Sera of mice immunised with the control strain LjTT also reacted weakly with human IgE but according to a Student's *t*-test reactivities to IgE were specific for sera immunised with either Lj α -IdscFv2 or Lj ϵ 4.

4. Discussion

We have previously described the use of *L. johnsonii* as a mucosal vaccine delivery vehicle. The cell surface proteinase PrtB isolated from *L. delbrueckii* subsp. *bulgaricus* was used as a model antigen as our previous studies had shown that PrtB was immunogenic after intragastric delivery of recombinant Lj [20]. In this study, Lj was engineered to express PrtB linked to either an anti-idiotypic single chain fragment variable (α -IdscFv2), or a C ϵ 4 mimotope (C ϵ 4 mim). Both the C ϵ 4mim and the binding site of α -IdscFv2 were recognised by the protective monoclonal anti-IgE antibody, BSW17 and were able to induce a systemic anti-human IgE antibody response after subcutaneous and intranasal immunisation.

The original antibody providing the starting point for the α -IdscFv2 was our previously isolated anti-idiotypic Fab (Fab α -Id-B43) which mimics the epitope on human IgE recognised by BSW17 [13]. Additionally, parenteral immunisation of rabbits using Fab α -Id-B43 was shown to induce high-affinity antibodies with the same characteristics as BSW17. In order to target an antibody molecule onto the surface of Lj, the binding site of Fab α -Id-B43 was constructed as a single chain fragment variable (α -IdscFv), because Gram-positive bacteria lack an oxidative site, where heavy and light chain of an Fab could assemble. However, there are many examples in the literature describing scFv's which were unstable or showed suboptimal binding properties [27–29]. In addition, it is also known, that the nature of the linker may have an influence on the correct folding of a scFv [30,31]. Thus, three different linkers were tested for optimal assembly of the variable heavy and light chain regions of Fab α -Id-B43. The linker of α -IdscFv2, bearing a β -turn motif on both ends [24], proved to be the most appropriate for cloning the anti-idiotypic scFv into PrtB as it was the only scFv recognised by both BSW17 and the rabbit anti- Fab α -Id-B43 (BSW17-like) antibodies. Interestingly, IgE Savazal only weakly reacted with the rabbit anti-human C ϵ 4mim serum. This might be explained by the fact that

anti-C ϵ 4 serum was made against a linear peptide of nine amino acids which carries a different conformational structure compared to the full-length native IgE molecule. This conformational difference might prevent the translation of anti-peptide reactivity into specific recognition of native IgE.

This is the first report of engineering gram-positive bacteria to express an anti-idiotypic scFv (α -IdscFv2) that can be used for active immunisation. Other recent reports have also described the use of Gram-positive bacteria as carriers for the expression of scFv proteins. Gunneriusson et al. [33] have reported the use of *Staphylococci* to surface display scFv antibodies in order to develop a whole-cell diagnostic device and for using as an alternative to filamentous phages in scFv libraries [32]. More recently, *Lactobacillus zeae* producing scFv antibodies, recognising the streptococcal antigen I/II were used to deliver passive immunity to *Streptococcus mutans* in a rat model of caries development [33]. However, in contrast to these studies we were interested to use the recombinant Lj for active immunisation.

Both recombinant bacterial clones (Lj α -IdscFv2 and Lj ϵ 4) induced after subcutaneous or intranasal immunisation a specific anti-IgE response. However, the magnitude of the anti-IgE response was strongly influenced by the route of immunisation and depended on the recombinant bacterial clone used. Thus, both recombinant bacterial clones (Lj α -IdscFv2 and Lj ϵ 4) were first tried by feeding mice intragastrically. In that case, an antibody response was induced against Lj but only low antibody titers against scFv and ϵ 4 suggesting that both proteins fragments were weakly immunogenic via the oral route. In this study, upon subcutaneous immunisation, the α -IdscFv2 expressing clone (Lj α -IdscFv2) induced a higher anti-IgE immune response than the C ϵ 4mim expressing clone (Lj ϵ 4) indicating that the α -IdscFv2 is more immunogenic than the small C ϵ 4 mim. However, after intranasal immunisation the opposite was the case. This is consistent with the data obtained by oral delivery of phage particles expressing the same antigens [34]. In this study, C ϵ 4mim expressing M13 bacteriophages also induced a higher anti-IgE titer than Fab α -Id-B43 expressing phages. These results might be explained by the more complex conformational structure of the anti-idiotypic antibody fragments in comparison to the C ϵ 4 mim. Correctly folded anti-idiotypic antibodies are therefore more dependent on the route of immunisation where different conditions prevail. The nasal route of immunisation is thought to be especially restrictive for antibody structures, as the nasal cavity is known to harbour proteases specific for antibody structures [35]. Such proteases, produced by resident aerobic bacteria [36], might lead to the digestion of α -IdscFv2. Thus, Poethke et al. [37] have shown the presence in various human secretions such as the nasal mucus of a proteolytic enzyme which reacts with the Fab portion of immunoglobulin G and inhibits the binding of IgG to its antigen.

Our results show the induction of an anti-IgE response after intranasal immunisation with recombinant Lj, expressing

IgE epitopes. The question remains however, whether these antibodies are protective and result in reduction of allergic symptoms in atopic patients. Immunisation of monkeys using soluble C ϵ 4mim or Fab α -Id-B43 have shown that both IgE epitopes behaved as surrogate antigens and induced an anti-IgE response able to inhibit an in vivo passive cutaneous anaphylaxis test [38]. Thus, it can also be expected that recombinant Lj bearing the same antigens on their surface would induce a qualitatively similar anti-IgE immune response preventing binding of IgE to the Fc ϵ RI, and consequently inhibit the sensitisation of the effector cells implicated in allergic disease.

Acknowledgements

We wish to thank Elsbeth Gautschi, Andreas Hofmann, Barbara Lulič and Cornelia Witschi for their excellent technical assistance. This work was supported by a research grant from Nestec Ltd. and by the Swiss National Science Foundation grant number 3200B0-100651/1.

References

- [1] Corne J, Djukanovic R, Thomas L, Warner J, Botta L, Grandordy B, et al. The effect of intravenous administration of a chimeric anti-IgE antibody on serum IgE levels in atopic subjects: efficacy, safety, and pharmacokinetics. *J Clin Invest* 1997;99:879–87.
- [2] Fahy JV, Fleming HE, Wong HH, Liu JT, Su JQ, Reimann J, et al. The effect of an anti-IgE monoclonal antibody on the early- and late-phase responses to allergen inhalation in asthmatic subjects. *Am J Respir Crit Care Med* 1997;155:1828–34.
- [3] Babu KS, Holgate ST. The role of anti-IgE therapies in the treatment of asthma. *Hosp Med* 2002;63:483–6.
- [4] Nayak A, Casale T, Miller SD, Condemi J, McAlary M, Fowler-Taylor A, et al. Tolerability of retreatment with omalizumab, a recombinant humanized monoclonal anti-IgE antibody, during a second ragweed pollen season in patients with seasonal allergic rhinitis. *Allergy Asthma Proc* 2003;24:323–9.
- [5] Chervinsky P, Casale T, Townley R, Tripathy I, Hedgecock S, Fowler-Taylor A, et al. Omalizumab, an anti-IgE antibody, in the treatment of adults and adolescents with perennial allergic rhinitis. *Ann Allergy Asthma Immunol* 2003;91:160–7.
- [6] Finn A, Gross G, van Bavel J, Lee T, Windom H, Everhard F, et al. Omalizumab improves asthma-related quality of life in patients with severe allergic asthma. *J Allergy Clin Immunol* 2003;111:278–84.
- [7] Lemanske Jr RF, Nayak A, McAlary M, Everhard F, Fowler-Taylor A, Gupta N. Omalizumab improves asthma-related quality of life in children with allergic asthma. *Pediatrics* 2002;110:e55.
- [8] Soler M. Omalizumab, a monoclonal antibody against IgE for the treatment of allergic diseases. *Int J Clin Pract* 2001;55:480–3.
- [9] Knutti-Müller JM, Stadler BM, Magnusson CM, de Weck CM. Human IgE synthesis in vitro. Detection with monoclonal antibodies. *Allergy* 1986;41:457–67.
- [10] Rudolf MP, Vogel M, Kricek F, Ruf C, Zurcher AW, Reuschel R, et al. Epitope-specific antibody response to IgE by mimotope immunization. *J Immunol* 1998;160:3315–21.
- [11] Scott JK, Smith GP. Searching for peptide ligands with an epitope library. *Science* 1990;249:386–90.
- [12] Barbas 3rd CF, Kang AS, Lerner RA, Benkovic SJ. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc Natl Acad Sci USA* 1991;88:7978–82.
- [13] Vogel M, Miescher S, Kuhn S, Zurcher AW, Stadler MB, Ruf C, et al. Mimicry of human IgE epitopes by anti-idiotypic antibodies. *J Mol Biol* 2000;298:729–35.
- [14] Kricek F, Ruf C, Rudolf MP, Effenberger F, Mayer P, Stadler BM. IgE-related peptide mimotopes. Basic structures for anti-allergy vaccine development. *Int Arch Allergy Immunol* 1999;118:222–3.
- [15] Zegers ND, Kluter E, van Der Stap H, van Dura E, van Dalen P, Shaw M, et al. Expression of the protective antigen of *Bacillus anthracis* by *Lactobacillus casei*: towards the development of an oral vaccine against anthrax. *J Appl Microbiol* 1999;87:309–14.
- [16] Grangette C, Muller-Alouf H, Goudercourt D, Geoffroy MC, Turneer M, Mercenier A. Mucosal immune responses and protection against tetanus toxin after intranasal immunization with recombinant *Lactobacillus plantarum*. *Infect Immun* 2001;69:1547–53.
- [17] Shaw DM, Gaerthe B, Leer RJ, Van Der Stap JG, Smittenaar C, Heijne Den Bak-Glashouwer M, et al. Engineering the microflora to vaccinate the mucosa: serum immunoglobulin G responses and activated draining cervical lymph nodes following mucosal application of tetanus toxin fragment C-expressing lactobacilli [In Process Citation]. *Immunology* 2000;100:510–8.
- [18] Maassen CB, Laman JD, den Bak-Glashouwer MJ, Tielen FJ, van Holten-Neelen JC, Hoogteijling L, et al. Instruments for oral disease-intervention strategies: recombinant *Lactobacillus casei* expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis. *Vaccine* 1999;17:2117–28.
- [19] Gilbert C, Atlan D, Blanc B, Portailier R, Germond JE, Lapierre L, et al. A new cell surface proteinase: sequencing and analysis of the prtB gene from *Lactobacillus delbruekii* subsp. *bulgaricus*. *J Bacteriol* 1996;178:3059–65.
- [20] Scheppeler L, Vogel M, Zuercher AW, Zuercher M, Germond JE, Miescher SM, et al. Recombinant *Lactobacillus johnsonii* as a mucosal vaccine delivery vehicle. *Vaccine* 2002;20:2913–20.
- [21] Platteeuw C, Simons G, de Vos WM. Use of the *Escherichia coli* beta-glucuronidase (gusA) gene as a reporter gene for analyzing promoters in lactic acid bacteria [published erratum appears in Appl Environ Microbiol 1994 Jun;60(6):2207]. *Appl Environ Microbiol* 1994;60:587–93.
- [22] Pantoliano MW, Bird RE, Johnson S, Asel ED, Dodd SW, Wood JF, et al. Conformational stability, folding, and ligand-binding affinity of single-chain Fv immunoglobulin fragments expressed in *Escherichia coli*. *Biochemistry* 1991;30:10117–25.
- [23] Huston JS, Mudgett-Hunter M, Tai MS, McCartney J, Warren F, Haber E, et al. Protein engineering of single-chain Fv analogs and fusion proteins. *Methods Enzymol* 1991;23:46–88.
- [24] Hennecke F, Krebber C, Pluckthun A. Non-repetitive single-chain Fv linkers selected by selectively infective phage (SIP) technology. *Protein Eng* 1998;11:405–10.
- [25] Tang Y, Jiang N, Parakh C, Hilvert D. Selection of linkers for a catalytic single-chain antibody using phage display technology. *J Biol Chem* 1996;271:15682–6.
- [26] Rudolf MP, Furukawa K, Miescher S, Vogel M, Kricek F, Stadler BM. Effect of anti-IgE antibodies on Fc epsilonRI-bound IgE. *J Immunol* 1996;157:5646–52.
- [27] Brinkmann U, Reiter Y, Jung SH, Lee B, Pastan I. A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. *Proc Natl Acad Sci USA* 1993;90:7538–42.
- [28] Reiter Y, Brinkmann U, Jung SH, Lee B, Kasprzyk PG, King CR, et al. Improved binding and antitumor activity of a recombinant anti-erbB2 immunotoxin by disulfide stabilization of the Fv fragment. *J Biol Chem* 1994;269:18327–31.
- [29] Reiter Y, Brinkmann U, Webber KO, Jung SH, Lee B, Pastan I. Engineering interchain disulfide bonds into conserved framework regions of Fv fragments: improved biochemical characteristics of recombinant immunotoxins containing disulfide-stabilized Fv. *Protein Eng* 1994;7:697–704.
- [30] Worn A, Pluckthun A. Stability engineering of antibody single-chain Fv fragments. *J Mol Biol* 2001;305:989–1010.

- [31] Robinson CR, Sauer RT. Optimizing the stability of single-chain proteins by linker length and composition mutagenesis. *Proc Natl Acad Sci USA* 1998;95:5929–34.
- [32] Gunneriusson E, Samuelson P, Uhlen M, Nygren PA, Stahl S. Surface display of a functional single-chain Fv antibody on staphylococci. *J Bacteriol* 1996;178:1341–6.
- [33] Kruger C, Hu Y, Pan Q, Marcotte H, Hultberg A, Delwar D, et al. In situ delivery of passive immunity by lactobacilli producing single-chain antibodies. *Nat Biotechnol* 2002;20:702–6.
- [34] Zuercher AW, Miescher SM, Vogel M, Rudolf MP, Stadler MB, Stadler BM. Oral anti-IgE immunization with epitope-displaying phage. *Eur J Immunol* 2000;30:128–35.
- [35] Kirkeby L, Rasmussen TT, Reinholdt J, Kilian M. Immunoglobulins in nasal secretions of healthy humans: structural integrity of secretory immunoglobulin A1 (IgA1) and occurrence of neutralizing antibodies to IgA1 proteases of nasal bacteria. *Clin Diagn Lab Immunol* 2000;7:31–9.
- [36] Rasmussen TT, Kirkeby LP, Poulsen K, Reinholdt J, Kilian M. Resident aerobic microbiota of the adult human nasal cavity. *Apmis* 2000;108:663–75.
- [37] Poethke R, Mader M, Zedler I, Beuche W, Felgenhauer K, Kolbus N. A putative enzyme from various secretions specifically inhibits antibody–antigen interactions. *J Immunol Methods* 1996;191:149–57.
- [38] Stadler BM, Zuercher AW, Miescher S, Kricek F, Vogel M. Mimotope and anti-idiotypic vaccines to induce an anti-IgE response. *Int Arch Allergy Immunol* 1999;118:119–21.