

Oral anti-IgE immunization with epitope-displaying phage

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An essential requirement for oral vaccines is the ability to survive the harsh environment of the stomach in an antigenically intact form. As bacteriophages are adapted to this environment we used epitope-displaying M13 bacteriophages as carriers for an experimental oral anti-IgE vaccine. The feasibility of this approach was tested in a simulated gastric fluid using two different mimotopes as well as an anti-idiotypic Fab of the non-anaphylactogenic monoclonal anti-IgE antibody BSW17. All phage clones remained infective after this treatment. However, only epitopes displayed on the pVIII protein were still recognized by BSW17 whereas pIII-expressed epitopes were rapidly inactivated. Surprisingly, when used for oral immunization of mice all phage clones induced anti-IgE antibodies. In contrast, oral immunization with the purified, pVIII protein displaying the mimotope induced anti-phage but no anti-IgE antibodies. After feeding a single dose of mimotope-displaying bacteriophage, phage DNA could be detected in mouse feces for 10 days. Our results show that epitope-displaying bacteriophages can be used to induce an epitope-specific antibody response via the oral route.

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1 Introduction

A major limitation for the development of effective oral vaccines is the fact that the Ag has to remain antigenic under the harsh conditions in the stomach. Previous attempts to achieve this goal included the application of immunizing Ag in strong basic buffers (carbonate) to transiently neutralize the gastric fluid [1] or encapsulated in biodegradable microspheres, liposomes or ISCOMS (immune stimulating complexes) (reviewed in [2]). More recently, in the first edible vaccines, Ag were expressed as transgenes in plants [3]. An entirely new strategy, however, was presented by Delmastro et al. [4] using mimotope-expressing phages to immunize mice by the oral route. With different feeding protocols anti-phage and anti-mimotope antibody titers were induced. However, their findings suggested that immunization was even achieved with non-infective phage. Moreover no information was provided concerning the stability of their antigenic preparations.

Phage display technology has been increasingly used for the development of new passive and active vaccines. Antibodies of defined specificities can be selected from combinatorial antibody phage libraries [5] and used for passive immunization [6]. Recently great interest has focused on the use of anti-idiotypic antibodies (anti-Id) for active immunization to induce the corresponding idiotype antibody. This strategy has been successfully applied to induce antibodies against a mutant p53 [7], *Chlamydia trachomatis* [8] or streptococci [9]. Likewise, random peptide libraries have frequently been used to identify Ab-specific peptides (mimotopes) [10, 11]. Such mimotopes induced considerable antibody titers when used to immunize animals [12–14]. Similarly, mimotopes successfully mimicked the structure of Shigella carbohydrates [15] and DNA in animal models for SLE [16] and renal disease [17], indicating that mimotopes may be regarded as a new generation of subunit vaccines that allow the production of protective antibodies of defined specificity.

In this study we have compared an anti-idiotypic Fab and two different mimotopes of the monoclonal anti-IgE antibody BSW17 expressed either on the surface of M13 bacteriophage or as purified subunits as experimental oral anti-IgE vaccines. All phages retained their infectiv-

[1 19605]

Abbreviations: SGF: Simulated gastric fluid GI: Gastro-intestinal

ity when treated with a simulated gastric fluid (SGF) [18]. However, mimotopes and Fab expressed on the pIII protein of M13 were susceptible to digestion with SGF and lost their antigenicity within a few seconds. Nevertheless, all phages induced anti-phage and anti-IgE antibodies in mice fed at weekly intervals. Therefore, we speculate that orally administered phages might be able to transiently colonize *E. coli* of the enteric flora and re-express the immunogenic mimotopes or Fab. This hypothesis is supported by the fact that phage DNA could be re-isolated and amplified by PCR from mouse feces for 10 days after feeding a single dose of phage.

2 Results

2.1 Stability of immunizing Ag in an *in vitro* gastric simulation assay

We used different Fab- or peptide-phage clones and a purified mimotope-displaying phage protein (Fig. 1), recognized by the same monoclonal anti-IgE antibody termed BSW17, to induce BSW17-like anti-IgE antibodies via the oral route. To analyze the effects of the harsh conditions prevailing in the stomach we tested our Ag in a gastric simulation test. In this assay [18] samples are treated with SGF, a buffer at pH 1.2 containing a physiologic amount of pepsin (0.3). All phages carrying

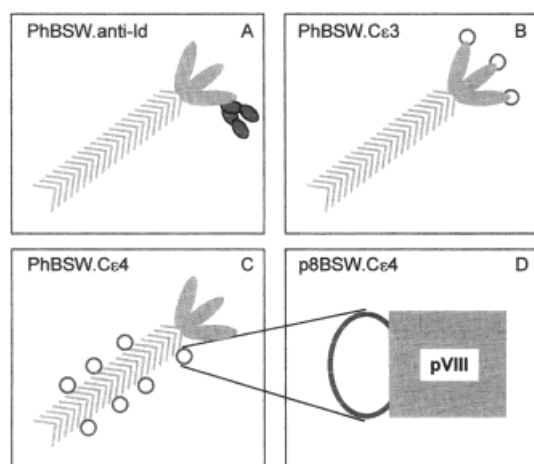


Fig. 1. Phage-displayed Ag used for oral immunization. All Ag are recognized by the monoclonal anti-IgE antibody BSW17. (A) anti-idiotypic Fab-phage clone. (B) 10mer peptide displayed as a fusion-protein with pIII of M13. This peptide is homologous to a part of the C ϵ 3-domain of IgE. (C) 9mer peptide displayed as a fusion-protein with pVIII of M13. This peptide is homologous to part of the C ϵ 4-domain of IgE. (D) Isolated pVIII protein of M13 displaying the same peptide as shown in (C).

Fab or peptide (Fig. 2 A, B, C) as well as control VCS M13 helper phage (Fig. 2 D) remained infective after treatment with SGF for up to 1 h. We observed a slight reduction of infectivity of PhBSW.C ϵ 3 (Fig. 2 B) but nevertheless a significant amount of phage remained infective. Using SDS-PAGE and Western blotting we found that p8BSW.C ϵ 4 was not digested by SGF (data not shown).

As a second parameter we analyzed the influence of SGF-treatment on the immunologic reactivity of the phage preparations. For this purpose an immunodot assay was performed using BSW17 as the catching antibody, and horseradish peroxidase (HRP)-labeled anti-phage antibody for detection of bound phage protein. As shown in Fig. 2 E and F, PhBSW.anti-Id and PhBSW.C ϵ 3 were not recognized by BSW17 after less than 1 min of treatment with SGF, indicating that the antigenic determinants were no longer intact. In contrast, PhBSW.C ϵ 4 and p8BSW.C ϵ 4 were still recognized by BSW17 after

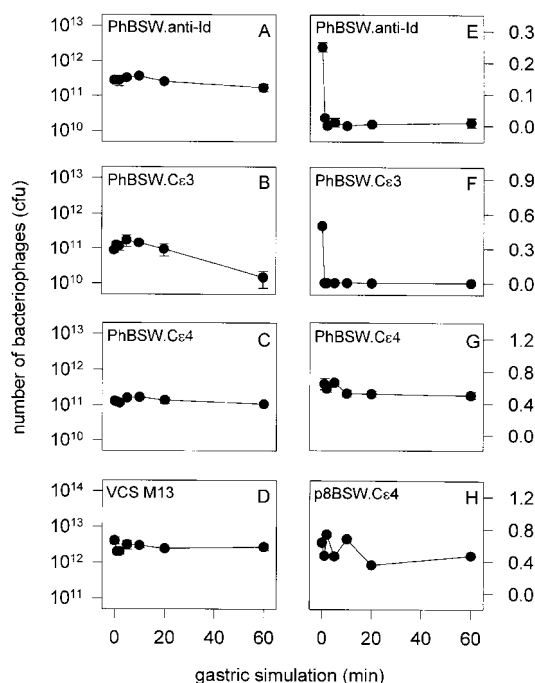


Fig. 2. Stability of immunizing Ag in an *in vitro* gastric simulation assay. (A)–(D) Infectivity. Phages were maintained in SGF for the time indicated. Infectivity was tested by infection of *E. coli* XL-1 blue and titration on agar petri dishes. cfu: colony forming units. (E)–(H) Immunologic reactivity. Phages or p8BSW.C ϵ 4 were treated with SGF. Immunologic reactivity was tested in an immunodot assay. BSW17 was used as catching antibody and bound phages of p8BSW.C ϵ 4 were detected with HRP-labeled anti-phage antibody. Relative OD (rel OD) was measured using a Gretag densitometer. Mean of duplicate values $\pm$ SD is shown.

60 min of treatment with SGF. No effect on infectivity nor immunologic reactivity was observed when the phages and p8BSW.Cε4 were treated with SGF without pepsin. In conclusion these data indicate that the pIII-displayed peptides but not the pVIII-displayed peptides were sensitive to digestion with gastric enzymes.

2.2 Oral immunization of mice with phages or p8BSW.Cε4

Subsequently all four preparations were used for oral immunization. Mice were pipette-fed with 10^{12} – 10^{13} phages or 100 µg p8BSW.Cε4 in 200 µl PBS at weekly intervals. Sera collected at different time points were tested for the presence of IgG antibodies induced against the carrier, i.e. the phage particles, in an immunodot assay. As shown in Fig. 3 A–D all phage preparations as well as p8BSW.Cε4 induced a strong anti-phage immune response. Nevertheless, large individual variations were observed.

To test whether the phage-displayed epitopes induced the production of antibodies similar to BSW17, mouse sera were analyzed for IgG anti-human IgE antibodies by

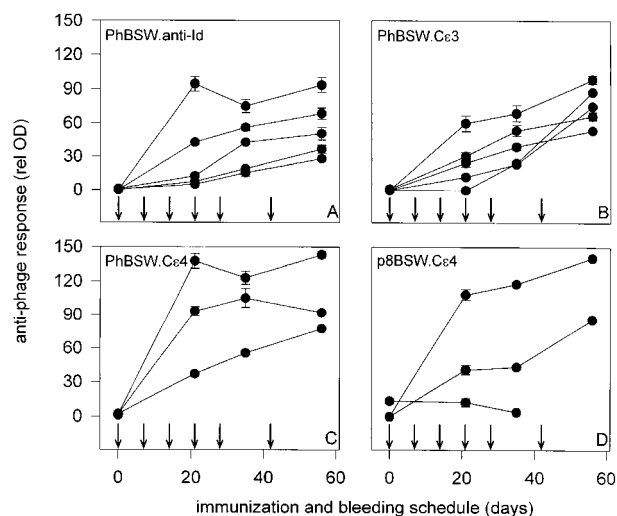


Fig. 3. Anti-phage antibodies in sera of orally immunized mice. Mice were fed at weekly intervals with 10^{12} – 10^{13} phages or 100 µg p8BSW.Cε4. Time points of oral immunization are indicated by arrows. Sera were analyzed in an immunodot assay. VCS M13 helper phage was dotted and sera (1:1000 in TBS-C) incubated overnight. Strips were developed with HRP-labeled anti-mouse IgG antibody. OD was measured with a Gretag densitometer. One line represents values obtained for an individual mouse (mean of duplicate values $\pm$ SD). Data from one of three similar experiments are shown.

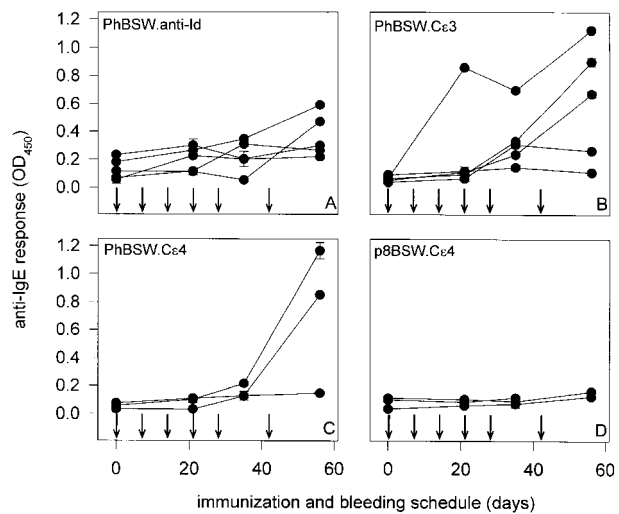


Fig. 4. Anti-IgE antibodies in sera of orally immunized mice. Mice were fed at weekly intervals with 10^{12} – 10^{13} phages or 100 µg p8BSW.Cε4. Time points of oral immunization are indicated by arrows. Sera were analyzed by ELISA. Half-well area flat-bottom EIA plates were coated with human IgE-Savasal (10 µg/ml). Sera (1:100 in TBS-C) were incubated over night and developed with HRP-labeled anti-mouse IgG antibody. One line represents values obtained for an individual mouse (mean of duplicate values $\pm$ SD). Data from one of three similar experiments are shown.

ELISA. As shown in Fig. 4 A, B and C sera of mice immunized with the three different phage preparations bound to human IgE, indicating that indeed anti-IgE antibodies had been induced. These findings are surprising given the fact that PhBSW.anti-Id and PhBSW.Cε3 lost their reactivity to BSW17 by treatment with SGF (Fig. 2 E and F). In contrast, oral immunization with p8BSW.Cε4 did not induce any detectable anti-IgE activity even though p8BSW.Cε4 displays the same peptide as PhBSW.Cε4 (Fig. 4 D). Similar results were obtained when the same sera were analyzed using another type of ELISA plate. As a control a strain of *E. coli* transfected with K88 fimbriae and expressing LTB was infected with PhBSW.Cε3 and then fed to mice following the same oral immunization protocol. This treatment induced only very low titers of anti-IgE antibodies, probably due to the very low initial number of phage (10^8 phage-infected bacteria vs. 10^{12} – 10^{13} phages). We conclude that the extremely low number of living bacteria present in the purified phage preparations (around 10^2 /ml, unpublished observation) does not influence our oral immunization protocol.

Importantly, i.m. immunization of mice with the same four Ag in Freund's adjuvant yielded results similar to those obtained by oral immunization. Notably, the anti-

phage and anti-IgE antibody titers were in the same order of magnitude for oral and for i.m. immunization (data not shown). Sera were also analyzed for the presence of IgA antibodies against phage or human IgE, but no serum IgA against these antigens was detected before or after oral immunization.

2.3 Specificity of anti-IgE antibodies induced by oral immunization

To confirm the specificity of the induced anti-IgE antibodies, sera collected 56 days after the first immunization with PhBSW.anti-Id, PhBSW.Cε3 or PhBSW.Cε4 were used in a competition assay to inhibit the binding of BSW17 to IgE-SUS11. Pre-immune sera of the same mice were used as controls. As shown in Fig. 5 all three sera successfully competed with BSW17 for binding to IgE, indicating that anti-IgE antibodies present in the mouse sera recognized the same epitope as BSW17. Nevertheless, only a 30–40 % inhibition of BSW17 binding was achieved (Fig. 5B). In contrast, serum of p8BSW.Cε4-immunized mice did not show any inhibition (data not shown).

The same sera were assessed in an immunodot assay for their binding to chemically synthesized, KLH-coupled

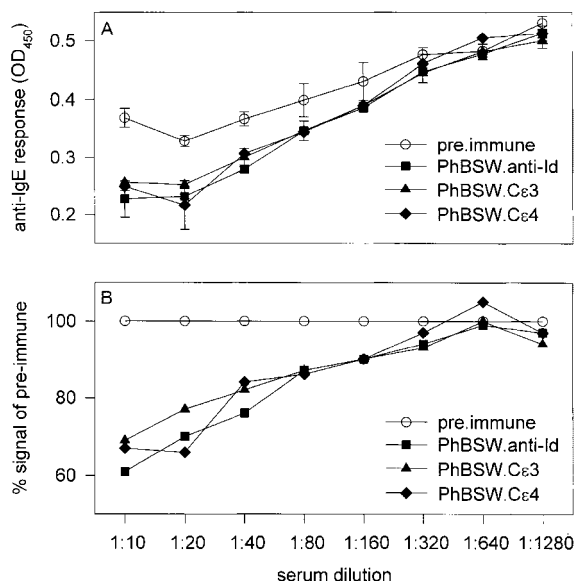


Fig. 5. Specificity of anti-IgE antibodies. IgE-SUS11-coated ELISA plates were incubated for 4 h with HRP-labeled BSW17 (1:20 000 in TBS-C) mixed with various amounts of three individual mouse sera collected at day 56 after first immunization. Results are expressed as (A) A_{450} and (B) % inhibition of the signal measured for pre-immune serum. Data from one of four similar experiments are shown.

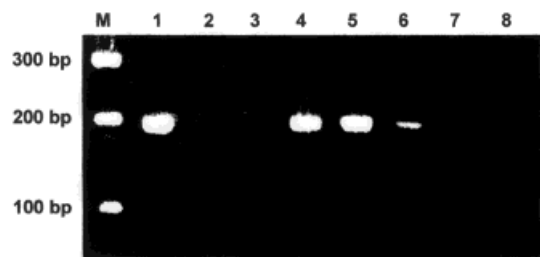


Fig. 6. PCR analysis of DNA isolated from fecal bacterial cultures from mice orally immunized with PhBSW.Cε3. Feces of mice were enriched for phage-infected, gram-negative bacteria by culture in MacConkey broth containing amphotericin B and tetracycline. Plasmid DNA was isolated from bacterial pellets and used as the template in a PCR reaction with primers specific for a DNA fragment of PhBSW.Cε3. Templates used for PCR: M: Marker (100 bp ladder); lane 1: purified DNA of PhBSW.Cε3 (positive control); lane 2: no template DNA (negative control of the PCR reaction); lane 3: fecal DNA isolated at day 0 of immunization (negative control); lane 4: day 3; lane 5: day 7; lane 6: day 10; lane 7: day 14; lane 8: day 21.

peptides derived from PhBSW.Cε3 and PhBSW.Cε4. The sera collected at day 56 specifically reacted with these synthetic peptides whereas the pre-immune sera did not bind (data not shown). This is a further indication that the antibodies induced by oral immunization resembled BSW17.

2.4 PCR-detection of PhBSW.Cε3-DNA re-isolated from mouse feces

To assess the persistence of PhBSW.Cε3 in bacteria of the gastrointestinal (GI) tract, we isolated plasmid DNA from feces of mice that had been immunized with a single dose of PhBSW.Cε3. This DNA was used as a template to perform a PCR using primers specific for a fragment of PhBSW.Cε3 DNA. As shown in Fig. 6, PhBSW.Cε3-DNA was detected from days 3 to 10 after a single immunization. These data demonstrate that phages transiently persisted in the GI tract of mice. However, the PCR protocol applied here only allows a qualitative analysis as opposed to quantitative or semi-quantitative PCR and therefore no conclusion on the amount of phage present in feces can be drawn.

3 Discussion

Our results show that phages displaying different IgE epitopes or anti-idiotypic Fab fragments recognized by the monoclonal anti-IgE antibody BSW17 can be used

for oral immunization. All phage particles, expressing either IgE mimotopes or anti-idiotypic Fab fragments on the pIII or pVIII protein, induced a specific anti-IgE response. In contrast the purified pVIII protein displaying the same mimotope as one of the intact phages did not induce anti-IgE antibodies. PCR analysis of phage DNA re-isolated from mouse feces indicated a transient persistence of the phage in the GI tract for up to 10 days.

Fab or mimotopes linked to the pIII were not stable in the gastric simulation test. However, when used for oral immunization these phages induced anti-IgE antibodies. This apparent paradox can be explained in different ways. Phages may infect gram-negative bacteria of the GI tract (*E. coli*) and re-express the antigenic determinants as new phage is produced. According to our data, infection of the gram-negative flora of the GI tract may well occur, as phages treated with SGF remain infective (a finding not surprising given the fact that acid is routinely used for elution of phages during selection procedures), and as naturally occurring *E. coli* were shown to carry and express F-plasmids [19, 20]. Furthermore, our finding that after a single inoculation of M13 in mice, phage DNA can be re-isolated between days 3 to 10 from feces further supports this hypothesis. Nevertheless, it may be speculated that phages only adhere non-specifically to structures of the GI tract, resulting rather in persistence than in colonization. From our data it cannot be clearly concluded which of these hypotheses is true. However, the fact that also pIII-displayed mimotopes and Fab – though digested in the stomach – induced anti-IgE antibodies, may indicate that indeed infection followed by re-expression of the mimotope/Fab had occurred. Finally, it can be speculated that the *in vitro* gastric simulation test does not correctly mimic the effects of the stomach fluid, which contains more enzymes than pepsin alone. However, the use of this assay is generally approved for the analysis of (food) protein stability [21] and we therefore assume that this assay has a high predictive value.

Our conclusion that during oral immunization with phages infection of the enteric flora occurs seems to be in contrast to the report of Delmastro et al. [4]. In this study it was shown that UV-inactivated phages could also be used for oral immunization. Our own experiments have shown that treatment with UV does not inactivate phages completely (data not shown) and it may thus be speculated that a small number of infective phages might be sufficient to transiently populate the enteric flora. Importantly, in their report only the anti-phage response induced by UV-inactivated phages is shown. This finding is in line with our data, demonstrating that even oral immunization with the purified coat-protein of M13 (p8BSW.Cε4) induces anti-phage antibodies. How-

ever, no further experiments have been presented in the above-mentioned report that assess whether only infective phages induce anti-mimotope antibodies.

As shown in Fig. 5 competition of immune sera with HRP-labeled BSW17 resulted in 30–40 % inhibition of BSW17 binding. This incomplete inhibition may be due to several reasons. First, it was shown, that BSW17 once bound to IgE dissociates with a very low off-rate [22] and thus, complete inhibition would only be observed using a large excess of competitor. Secondly, a 1:20 000 dilution with HRP-labeled BSW17 corresponds to about 100 ng/ml BSW17. As only small amounts of anti-IgE antibodies were induced by oral immunization with phage the amount of anti-IgE may be insufficient to achieve a more effective inhibition. PhBSW.Cε4 and p8BSW.Cε4 display the same peptide and both were shown to be stable in the SGF test. But only oral immunization with PhBSW.Cε4 induced anti-IgE antibodies. The most probable explanation for these contrasting results are the different manners in which PhBSW.Cε4 and p8BSW.Cε4 are presented to the immune system. As a phage particle the pVIII protein subunits are highly organized and many mimotopes (30–100 per phage) are presented in series. In contrast to this we observed that the spontaneously forming aggregates of p8BSW.Cε4 dissociated in SGF. Thus the structural presentation of the mimotopes seems to be critical for the induction of anti-mimotope antibodies. These findings are in accordance with reports of Bachmann et al. [23, 24], showing that induction of an efficient anti-viral antibody response was dependent on the highly structured, repetitive organization of the viral surface Ag.

The interest in the anti-IgE model system we have used in this study has recently increased due to its potential clinical relevance. Currently the only widespread non-palliative treatment for IgE-mediated allergies is immunotherapy, using increasing doses of the allergenic proteins to desensitize the patient [25]. In the last few years it was shown that neutralizing, non-anaphylactogenic anti-IgE antibodies can be used to attenuate the effects of IgE in allergic patients (reviewed in [26]). The monoclonal anti-IgE antibody BSW17 was previously also shown to have these advantageous characteristics (reviewed in [27]) and a humanized anti-IgE antibody with characteristics similar to BSW17 has been tested in phase I [28] and phase II [29, 30] clinical studies with very promising results. However, the beneficial effects of this passive antibody treatment are only achieved if relatively large amounts of antibody are applied, making this treatment very expensive. Thus, it would be an advantage to replace or combine the passive treatment with an active immunization, using a mimotope or an anti-IgE antibody against BSW17-like antibodies. Our report demonstrates

that active immunization with phage particles can be achieved via the oral route given a powerful delivery system. However, it may be argued that the data obtained in mice cannot be applied for the human system as human IgE mimotopes represent an exoantigen for mouse but an autologous protein for the human. Preliminary data obtained in a rhesus monkey model, which is close to the human system as the Fc ϵ RI of monkey mast cells can bind to human IgE, indicate that the mimotopes and anti-IgE used in this study induced an effective and protective anti-human IgE response as shown by abrogation of passive cutaneous anaphylaxis by mimotope immunization (F. Kricek, Novartis, Vienna, personal communication). These findings indicate that IgE mimotopes represent structures that are foreign enough to the immune system to induce an immune response but nevertheless similar enough to human IgE that the antibodies produced recognize human IgE. Our data demonstrate that both pIII- and pVIII-displayed epitopes when used for oral immunization induce a humoral immune response even though the fate of the peptides/Fab in the stomach is probably distinct. However, more efforts will be required to establish the optimal formulation of an active immunization for the induction of beneficial anti-IgE antibodies. Using phages as carriers for oral immunization quite a large number of immunizations was needed to evoke an Ab-response (six immunizations in this study; similar or even higher numbers by others [4]). On a molar basis the amount of phage used (10^{12} – 10^{13}) corresponds to as little as 60–2400 ng peptide or Fab, which might explain why the rather large number of immunizations was needed (calculations based on a molecular weight of 40 kDa for Fab and 1 kDa for peptide and the assumption of monovalent expression). To circumvent this limitation we are currently trying to express mimotopes in higher density on carriers that are by themselves less immunogenic, *i.e.* on the surface of bacteria belonging to the natural enteric flora.

4 Materials and methods

4.1 Animals and reagents

Female 6–8-weeks-old BALB/c mice from the animal facilities of the University of Bern, Switzerland were used throughout the whole study. BSW17 is a monoclonal anti-IgE antibody described earlier [31]. Polyclonal anti-phage antibody was produced in our laboratory [32]. IgE-Savasal was kindly provided by Dr. V. Savasal (Pilsen, Czech Republic). IgE-SUS11 is a monoclonal human IgE hybridoma [33]. HRP-labeled goat anti-mouse IgG antibody and sheep anti-mouse IgG antibody was purchased from Cappel (ICN Bio-medical GmbH, Eschwege, Germany).

4.2 Ag and immunization

Three different phage clones and a purified subunit from one of those phage clones were used for oral immunization as shown in Fig. 1. All four preparations have in common that they display epitopes that are recognized by BSW17.

PhBSW.anti-IgE is an anti-idiotypic Fab directed against the Ag-binding site of BSW17 (Vogel M. et al., manuscript on the isolation and molecular characterization in preparation) expressed as a fusion protein with the pIII protein of phage M13. PhBSW.C ϵ 3 is a decapeptide homologous to the third domain of human IgE recognized by BSW17 (VNLPWSFGLE, Rudolf M.P. et al., manuscript submitted) expressed as a fusion protein with the pIII protein of M13. PhBSW.C ϵ 3 was isolated from the random peptide library am3–4 [34]. PhBSW.C ϵ 4 is a cysteine-constrained nonapeptide (CRRHNYGFWVC) described in detail elsewhere [14]. This peptide was isolated from a random peptide library (provided by F. Felici, Instituto Ricerche di Biologia Molecolare, Rome, Italy) where peptides are expressed as fusion proteins with the pVIII protein of M13 [7]. This peptide contains a structural motif similar to a part of the C ϵ 4 domain of the IgE molecule and is also specifically recognized by BSW17 [14]. p8BSW.C ϵ 4 is the purified pVIII-peptide fusion protein of PhBSW.C ϵ 4. This protein was produced according to a modified protocol for phage amplification and purified by precipitation using 55 % ammonium sulfate (Fluka Chemie AG, Buchs, Switzerland).

For oral immunization one dose contained either 200 μ l phages (10^{12} – 10^{13}) in PBS or 100 μ g p8BSW.C ϵ 4 in PBS applied at weekly intervals (day 0, 7, 14, 21, 28 and 42). Doses were applied by pipette-feeding using soft plastic Pasteur pipettes (Liquipettes Elkay, Roth AG, Rheinach, CH).

4.3 *In vitro* gastric simulation assay

Resistance of the immunizing Ag to digestion in the stomach was analyzed in a gastric simulation assay as described earlier [18]. Briefly, phages or p8BSW.C ϵ 4 were maintained in SGF containing 0.32 % (w/v) pepsin (Fluka), 0.03 M NaCl at pH 1.2 (adjusted with HCl). At different time points (Fig. 2) aliquots of 20 μ l were removed and neutralized with 13 μ l 160 mM Na₂CO₃. To determine the titer of remaining infective phage particles, fresh *E. coli* XL-1 blue were infected for 15 min, and aliquots titrated on agar plates containing appropriate antibiotics. The effect of the acid treatment on the immunologic reactivity of the immunizing Ag was analyzed in an immunodot assay [35]. Nitrocellulose strips (Millipore Corp., Bedford, MA) were coated with 1 μ l BSW17 (500 μ g/ml) and blocked with TBS containing 1.5 mg/ml casein (Fluka; TBS-C). Aliquots of acid-treated phages were incubated overnight and detected with polyclonal HRP-labeled anti-phage antibody (1:1000 in TBS-C) for 4 h. Strips were developed as described [32]. The OD of the dots

was measured using a hand-held Gretag densitometer (Gretag Limited, Regensdorf, Switzerland) and are expressed as relative OD (rel OD) (see [32, 35]).

4.4 Analysis of mouse sera for anti-phage and anti-IgE antibodies

Sera of orally immunized mice were collected at the indicated time points (Fig. 3, Fig. 4) by retro-orbital bleeding. To determine mouse IgG anti-phage antibodies an immunodot was performed. Nitrocellulose strips were coated with VCS M13 helper phage and blocked with TBS-C. Sera were diluted 1:1000 in TBS-C and incubated overnight, bound antibody detected with HRP-labeled goat anti-mouse antibody (1:1000 in TBS-C) for 4 h, and developed as described in Sect. 4.3.

Mouse IgG anti-human IgE activity in mouse sera was measured by ELISA. Half-area flat-bottom EIA/RIA plates (Costar, 3690, Integra Biosciences, Wallisellen, Switzerland) were coated with 10 µg/ml IgE-Savasol. After blocking with TBS-C, mouse sera (75 µl; 1:100 in TBS-C) were incubated overnight. Thereafter, HRP-labeled goat anti-mouse antibody (1:1000 in TBS-C) was added for 4 h. Plates were developed with 3,3',5,5'-tetramethyl-benzidine (Fluka)/H₂O₂ and absorbance was measured at 450 nm.

4.5 Competition assay

IgE-SUS11 coated Immulon II ELISA plates (Dynex Technologies, Inc., Chantilly, VA) were incubated with HRP-labeled BSW17 (1:20 000 in TBS-C) mixed with various amounts of different mouse sera collected before immunization (pre-immune) and at day 56 (as indicated in Fig. 5) for 4 h. Plates were developed with TMB/H₂O₂. Results are expressed as A₄₅₀ and as % inhibition of the signal measured for pre-immune serum.

4.6 Analysis of PhBSW.Cε3 DNA re-isolated from mouse feces

Gram-negative bacteria potentially infected with phages were enriched from mouse feces by culture in MacConkey broth (Difco, Chemie Brunschwig, Basel, Switzerland) containing 2.5 µg/ml amphotericin B (Fluka) and 20 µg/ml tetracycline. Plasmid DNA from 60 ml culture was purified using a commercial kit (Nucleobond, Macherey-Nagel, Oensingen, Switzerland). A PCR was performed using the primers FK344n (CTGTAGCATTCACAGACAAC) and FK350n (TCTCACTCGGCCGACGGGGCT). FK344n is specific for M13 DNA, FK350n for the framework of the random oligonucleotide defining the sequence of the mimotope. As a negative control we used DNA isolated from feces of non-immunized mice, as a positive control purified phage DNA from PhBSW.Cε3 phage.

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