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Comparison of the immunogenicity of BM32, a recombinant hypoallergenic B cell epitope–based grass pollen allergy vaccine with allergen extract–based vaccines

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To the Editor

Allergen-specific immunotherapy (AIT) is a clinically and cost-effective allergy treatment that modifies the course of the disease and has long-lasting effects.^{1,E7,E8} However, allergen extract–based forms of AIT require administration of multiple doses, which makes treatment cumbersome and leads to poor compliance in patients.² A number of approaches were proposed to address this issue, including use of AIT materials with higher safety, such as allergoids, recombinant allergen derivatives, and allergen-derived peptides, which allow shortening of the buildup phase.^{E9}

In this study we have investigated the ability of the recombinant B cell epitope–based allergy vaccine BM32 to induce allergen-specific IgG antibodies and the ability of these antibodies to inhibit allergic patients' IgE binding to grass pollen allergens, as well as allergen-induced T-cell proliferation. BM32 is a recombinant grass pollen (*Phleum pratense*) allergy vaccine based on 4 fusion proteins consisting of peptides from the 4 major timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5, and Phl p 6) fused to the PreS carrier protein from hepatitis B, which lacks relevant allergenic activity^{E10} and therefore can be injected into allergic patients without need for up dosing.^{3,4} For our immunogenicity studies, we have used a dose (ie, 20 µg of each of the 4BM fusion proteins) that has been safely administered to allergic patients (ClinicalTrials.gov identifiers NCT01445002, NCT01538979, and NCT02643641).⁴

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The main finding of our current study was that 3 monthly subcutaneous injections of aluminum hydroxide-adsorbed BM32 induced IgG antibody levels to the major grass pollen allergens Phl p 1, Phl p 5, and Phl p 6 in rabbits, which were comparable with natural allergen extract-based registered grass pollen allergy vaccines requiring more than 8 injections (Allergovit grass; Allergopharma, Reinbek, Germany; Alutard SQ grass mix; ALK-Abelló, Hørsholm, Denmark; and Phostal grasses + rye; Stallergenes, Antony, France; see the Methods section in this article's Online Repository at www.jacionline.org; Fig 1), whereas almost no response was observed with Pollinex (Pollinex Quattro Plus grasses + rye; Bencard Allergie GmbH, Munich, Germany), a vaccine based on 4 injections. Importantly, BM32 induced higher levels of Phl p 2-specific IgG antibodies than any of the registered allergen extract-based vaccines (Fig 1). We consider this an important finding because it has been shown that group 2 allergens are major grass pollen allergens recognized by more than 60% of patients with grass pollen allergy and, when compared with the other grass pollen allergens by using skin testing, were found to show high allergenic potency.⁵ Thus our results indicate that it should be possible to build up sufficient levels of grass pollen allergen-specific IgG responses with only few injections (ie, 3-5) of BM32, whereas traditional allergy vaccines require more than double the number of up dosing injections. Sublingual treatment even requires daily administration. Therefore we think that the treatment schedules based on BM32 will be more convenient for patients and should increase their compliance.

The kinetics of allergen-specific IgG antibodies induced with BM32 were similar to those induced by natural allergen extract-based vaccines in terms of increases and decreases (Fig 1). According to the hapten carrier principle described by the Nobel laureate B. Benacerraf,¹ a robust antibody response can be obtained against a haptenic structure lacking T-cell epitopes if this structure is covalently bound to an unrelated carrier molecule, such as the PreS that contains T-cell epitopes. Carrier-specific T cells support antibody production against the carrier and the hapten. Therefore the question was whether an allergen-specific IgG immune response initiated with BM32 can be boosted by repeated immunization with BM32. To address this question, we kept rabbits that had received a first course of 3 monthly injections with BM32 for 5.5 months without immunization and then applied 1 booster injection; after an additional 4.5 months, we administered another booster injection (see Fig E1 in this article's Online Repository at www.jacionline.org). BM32-induced grass pollen allergen-specific IgG production could be readily boosted after it had decreased, indicating that it will be possible to boost allergen-specific IgG levels in several consecutive pollen seasons (see Fig E1). We also found that rabbits immunized with BM32 produced PreS-specific antibodies (data not shown), and PreS-specific antibodies were also induced in patients who were treated with BM32.^{4,6} Interestingly, PreS-specific antibodies induced through vaccination with BM32 also protected liver cells from hepatitis B infections.⁶

Another important observation was that BM32-induced IgG antibodies not only reacted with the 4 recombinant timothy grass pollen allergens but also with natural timothy grass pollen allergens, and we observed cross-reactivity with the major allergens in other grass species belonging to the Pooideae and Chloridoideae using immunoblotting (see the Methods section and Fig E2 in this article's Online Repository at www.jacionline.org). This finding

suggests that the BM32-induced antibodies might also recognize allergens from other grass species.

It is known that induction of allergen-specific IgG antibodies that block IgE binding to allergens in allergic patients is important for the clinical efficacy of AIT because such blocking IgG inhibits allergen-induced mast cell and basophil degranulation and IgE-facilitated allergen presentation and the consecutive T-cell activation.⁷ Therefore we compared BM32-induced IgG with IgG antibody levels induced with allergen extract-based vaccines regarding their ability to inhibit IgE binding to the major timothy grass pollen allergens and natural timothy grass pollen allergen extract in allergic patients using an IgE inhibition ELISA (see the Methods section and Table E1 in this article's Online Repository at www.jacionline.org and Fig 2). These experiments showed that BM32-induced IgG inhibited IgE binding to Phl p 1, Phl p 5, and Phl p 6, as well as against timothy grass pollen extract, in allergic patients, although antibodies induced by Allergovit and Alutard inhibited IgE binding to timothy grass pollen extract significantly better than those induced by BM32 ($P = .0008$, Fig 2). Anti-Pollinex antibodies produced less than 10% inhibition of IgE binding to the allergens and timothy grass pollen extract and thus inhibited IgE binding to timothy grass pollen extract and the individual grass pollen allergens significantly less ($P = .0008$) than those induced by all the other vaccines. The induction of grass pollen allergen-specific IgG by 4 injections of Pollinex was also modest in allergic patients and only significant after the second treatment year.^{8,9}

In the IgE inhibition experiments BM32-induced IgG blocked IgE binding to Phl p 2 significantly better than IgG antibodies induced by the commercial vaccines ($P = .0008$, Fig 2). Therefore one might assume that BM32 could be superior to the extract-based vaccines regarding protection of group 2 allergen-induced symptoms. Finally, we also could demonstrate that BM32-induced antibodies inhibited specifically grass pollen allergen-induced T-cell proliferation (see the Methods section and Fig E3 in this article's Online Repository at www.jacionline.org), which suggests that BM32 might also have beneficial effects on T cell-mediated late-phase symptoms of grass pollen allergy. However, a limitation of our study is that we performed the immunogenicity study in naive animals, and therefore there might be differences regarding already sensitized patients.

In summary, our immunogenicity studies indicate that induction of grass pollen allergen-specific protective antibody responses requires considerably fewer injections of BM32 compared with natural allergen extract-based vaccines and that the use of carrier proteins in the vaccine helps to overcome the poor immunogenicity of group 2 allergens. Given the strong reduction of allergenic activity of BM32, one might expect that BM32 represents a grass pollen allergy vaccine that could be superior to allergen extract-based vaccines.

Methods

Recombinant allergens, allergen extracts, and products for subcutaneous AIT of grass pollen allergy

The purified recombinant grass pollen allergens Phl p 1, Phl p 2, Phl p 5, and Phl p 6 were obtained from Biomay AG (Vienna, Austria). Grass pollen from *Phleum pratense*, *Lolium*

perenne, *Cynodon dactylon*, and *Secale cereale* were purchased from Allergon (Välinge, Sweden), and aqueous pollen extracts were prepared.E1

Products for subcutaneous AIT of grass pollen allergy were purchased from 4 companies. The registered vaccines were obtained from the pharmacy of the Vienna General Hospital. The following products were used: Allergovit 006 (grasses, 100%; article no. 12142; expiration date, October 2011; reference no. 100904579; Charge batch: J0653373; Allergopharma, Reinbek, Germany); Alutard SQ L299 grass mix (batch: 008342708001; expiration date, November 2011; ALK-Abelló, Hørsholm, Denmark); Phostal 671 (rye 50% + 688 5 grasses 50%; batch: AT0071486/1490588; expiration date, July 2011; Stallergenes, Antony, France); and Pollinex Quattro Plus (1 mL [4130 grasses + rye], batch: 127758; reference no. 2010/050/003325; Bencard Allergie GmbH, Munich, Germany).

The products were stored at +4°C, as recommended by the manufacturers, and used during 2010, long before the end of the expiration dates. Treatment was performed exactly according to the recommendations of the manufacturers, as shown also in Fig 1, A.

Alutard SQ contains a mix of 6 natural grass pollen extracts (*Phleum pratense*, *Poa pratensis*, *Festuca pratensis*, *Lolium perenne*, *Dactylis glomerata*, and *Avena elatior*) adsorbed to aluminum hydroxide. Phostal is a vaccine composed of a 6-grass extract mix (*Phleum pratense*, *Poa pratensis*, *Lolium perenne*, *Dactylis glomerata*, *Anthoxanthum odoratum*, and *Secale cereale*) absorbed onto calcium phosphate. Allergovit contains a grass pollen allergoid composed of 6 different grass pollen species (*Phleum pratense*, *Poa pratensis*, *Festuca pratensis*, *Lolium perenne*, *Dactylis glomerata*, and *Holcus lanatus*) adsorbed to aluminum hydroxide. Pollinex is based on an allergoid made from extracts of 12 grass pollens and rye cereal (*Agrostis tenuis*, *Dactylis glomerata*, *Cynosurus cristatus*, *Arrhenatherum elatius*, *Poa pratensis*, *Festuca pratensis*, *Alopecurus pratensis*, *Lolium perenne*, *Bromus* species, *Anthoxanthum odoratum*, *Phleum pratense*, *Holcus lanatus*, and *Secale cereale*), which is adsorbed onto L-tyrosine with addition of the immunostimulatory adjuvant monophosphoryl lipid A.E2

Formulation of aluminum hydroxide–adsorbed vaccines containing BM32 fusion proteins or recombinant grass pollen allergens

An aluminum hydroxide–adsorbed vaccine containing 20 µg of each of the purified BM32 fusion proteins (BM321, BM322, BM325, and BM326) or each of the purified timothy grass pollen allergens (rPhl p 1, 20 µg; rPhl p 2, 10 µg; rPhl p 5, 20 µg; rPhl p 6, 10 µg) in a dose volume of 200 µL was obtained by using the following procedure: in a first step the individual fusion proteins were adsorbed to aluminum hydroxide (Alhydrogel; Brenntag Biosector, Ballerup, Denmark; 10 mg of Al³⁺/mL), resulting in 4 intermediate adsorbates containing 0.4 mg/mL of the single fusion protein and 3 mg/mL Al³⁺ in 2 mmol/L phosphate buffer (pH 7.4). These 4 intermediate adsorbates were then mixed in a 1:1:1:1 fashion, resulting in the BM32 vaccine mix containing 0.1 mg/mL of each BM32 fusion proteins or wild-type allergens and 3 mg/mL Al³⁺ in 2 mmol/L phosphate buffer (pH 7.4).

Immunization of rabbits and detection of grass pollen allergen-specific rabbit antibodies

Rabbits were immunized subcutaneously 3 times in monthly intervals with aluminum hydroxide adsorbed BM32 containing 20 µg (n = 2) or 40 µg (n = 2) of each BM component or with an equimolar recombinant grass pollen allergen mix containing 20 µg of Phl p 1, 10 µg of Phl p 2, 20 µg of Phl p 5, and 10 µg of Phl p 6 (Charles River, Kisslegg, Germany).

For comparison, rabbits were immunized subcutaneously with AIT vaccines containing natural allergen extracts from 4 major companies (n = 2 per vaccine; Allergovit grass, Allergopharma; Alutard SQ grass mix, ALK-Abelló; Phostal grasses + rye, Stallergenes; and Pollinex Quattro Plus grasses + rye, Bencard Allergie GmbH), as recommended by the manufacturer. For Allergovit 7, injections of increasing strength were administered in weekly intervals, followed by one maintenance injection after 2 weeks (Fig 1, A). For Alutard, the shortened initial therapy schedule II was applied, consisting of 11 injections within the first 8 weeks (Fig 1, A). In the case of Phostal, 13 injections were given within 12 weeks, and for Pollinex, 3 injections were given in a 2-week interval, followed by a fourth injection after a further 4 weeks (Fig 1, A). Thus the preseasonal treatments were completed for BM32, the mix of recombinant allergens, Allergovit, Alutard, and Pollinex after 8 weeks and for Phostal after 12 weeks. Serum samples were taken from the rabbits on the day of first immunization (pre-serum) and in monthly intervals. Grass pollen allergen-specific IgG responses were measured by means of ELISA in sera obtained before and at 1-, 2-, 3-, and 4-month (Phostal) intervals (Fig 1, B).

The BM32-immunized rabbits continued to be monitored and vaccinated to study whether their immune response can be boosted. Their allergen-specific IgG response was measured in monthly intervals for a total duration of 13 months (Fig E1). Booster injections were administered after 7.5 and 12 months, and the final serum sample was obtained 4 weeks after the last immunization (Fig E1).

Measurement of grass pollen allergen-specific immune responses

ELISA plates (Thermo Scientific Nunc, Roskilde, Denmark) were coated with 3 µg/mL of each of the grass pollen allergens rPhl p 1, 2, 5, or 6 to determine grass pollen allergen-specific antibody responses. Allergen-specific rabbit IgG responses were measured by diluting sera 1:500 by means of ELISA. Bound rabbit IgG was detected with 1:2000 diluted donkey anti-rabbit horseradish peroxidase-coupled IgG antibodies (NA 934; GE Healthcare UK Limited, Chalfont St Giles, United Kingdom). Color development was done with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid).E3

Analysis of cross-reactivity of rabbit sera to nitrocellulose-blotted grass pollen extracts

Grass pollen extracts (*Phleum pratense*, *Lolium perenne*, *Secale cereale*, and *Cynodon dactylon*) were separated by using 15% preparative SDS-PAGE (24 µg of extract/cm of gel) and blotted onto a nitrocellulose membrane.E4 Nitrocellulose strips were incubated with 1:1000 diluted rabbit sera and, for control purposes, with the corresponding preimmune sera. Bound rabbit antibodies were detected with a 1:3000 diluted iodine 125-labeled goat anti-rabbit antibody (PerkinElmer Life Science, Boston, Mass) and visualized by using autoradiography.E5

ELISA competition assay for analyzing inhibition of human IgE binding to natural and recombinant grass pollen allergens by BM32-specific IgG antibodies

The ability of rabbit antibodies to inhibit the binding of IgE from patients with grass pollen allergy to timothy grass pollen extract or each of the recombinant timothy grass pollen allergens (rPhl p 1, 2, 5, and 6) was examined in ELISA competition assays, as described previously. E6 ELISA plates were coated with 0.2 µg per well of timothy grass pollen extract or 0.1 µg per well of each of the recombinant allergens preincubated with 1:4 diluted rabbit sera obtained 2 months after the last injection (ie, 3.IS for BM32, Allergovit, Alutard, and Pollinex and 4.IS for Phostal) and, for control purposes, with 1:4 diluted sera from rabbits without any immunization. After washing, plates were incubated with 1:5 diluted sera from patients with grass pollen allergy. Sera (n = 8) were residual serum samples from patients with grass pollen allergy who had been characterized by case history, skin prick test responses, and measurement of grass pollen-specific IgE antibodies (Table E1). Sera were anonymized and used with approval of the ethics committee of the Medical University of Vienna (EK1641/2014). Bound human IgE antibodies were detected with horseradish peroxidase-coupled goat anti-human IgE antibodies diluted 1:2500 (KPL, Gaithersburg, Md). The percentage of inhibition of IgE binding achieved by means of preincubation with anti-sera was determined as follows:

$$\text{Percentage inhibition of IgE binding} = 100 - \text{ODI}/\text{ODP} \times 100,$$

where *ODI* and *ODP* represent extinctions measured after preincubation with the rabbit immune and preimmune serum, respectively.

Inhibition of human allergen-specific T-cell proliferation by BM32-specific IgG or grass pollen allergen-specific antibodies

PBMCs were isolated from heparinized blood samples of patients with grass pollen allergy by means of Ficoll density gradient centrifugation (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). PBMCs (2×10^5) were cultured in triplicates in 96-well plates (Nunc; Nalge Nunc International, Roskilde, Denmark) in 200 µL of serum-free Ultra Culture medium (BioWhittaker, Rockland, Me) supplemented with L-glutamine (10 mL of 200 mmol/L L-glutamine per liter; Sigma, St Louis, Mo), β-mercaptoethanol (5 mL of 50 mmol/L β-mercaptoethanol per liter, Sigma) and gentamicin (2 mL of 50 mg/mL per liter of medium, Sigma) at 37°C and 5% CO₂ in a humidified atmosphere. PBMCs were stimulated with a mix of rPhl p 1, 2, 5, and 6 (0.5 µg per well of each allergen, recombinant mix) or rBet v 1 (2 µg per well) as a control in the presence of heat-inactivated (56°C for 30 minutes) 1:10 diluted sera obtained from rabbits 1 month after the last immunization with BM32 (40-µg dose), Alutard, or Allergovit or from normal rabbits. For control purposes, 4 U of IL-2 per well (Boehringer, Mannheim, Germany) or medium alone was used.

Statistical analysis

For statistical analyses, GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif) was used. Differences between groups were compared by using the Wilcoxon signed-rank

test. For multiple comparisons, the Bonferroni-Holm correction was applied. *P* values of less than .05 were considered significant.

Extended Data

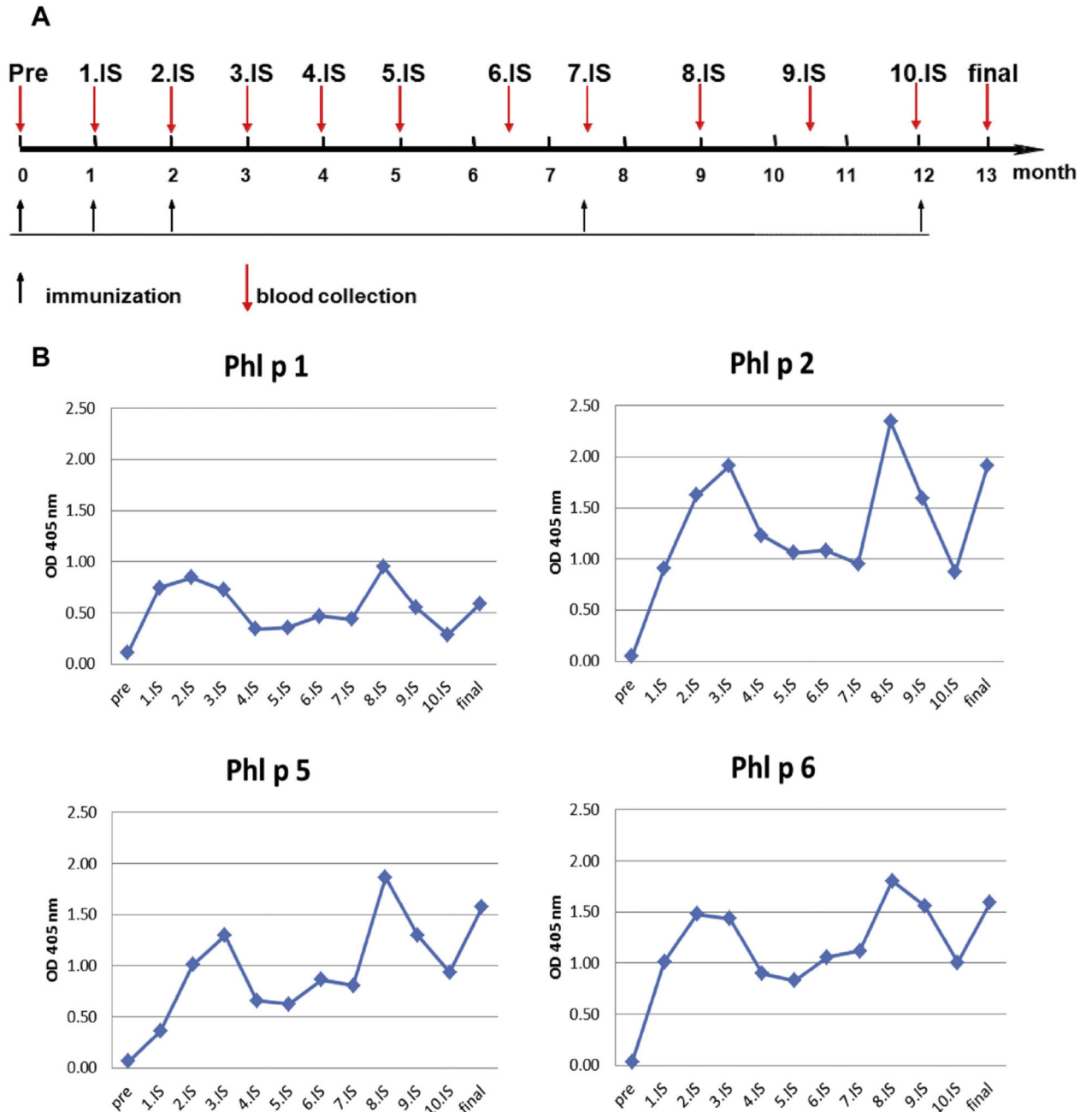


Fig E1.

Boosting of BM32-induced allergen-specific IgG antibody responses. **A**, Scheme and time course of boosting experiment. Indicated are the time course (*x-axis*), injections, and dates of serum collection. **B**, Kinetics of allergen-specific IgG responses in BM32-immunized

rabbits after initial immunization and 2 booster injections. Shown are mean OD values corresponding to allergen-specific IgG levels (*y-axes*) at different time points (*x-axes*).

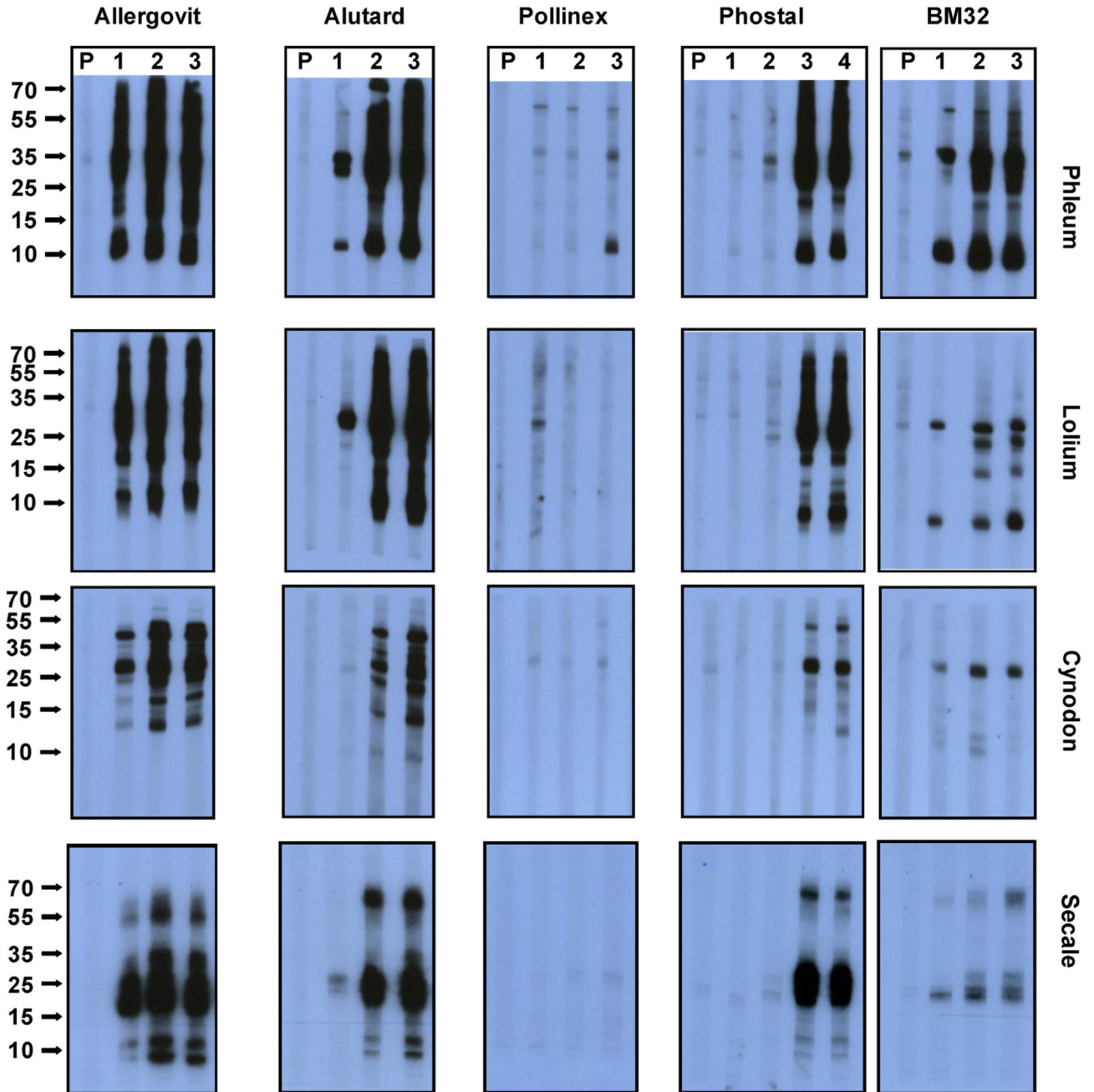


Fig E2. Cross-reactivity of BM32-induced antibodies with pollen extracts from different grasses. Nitrocellulose-blotted pollen extracts from *Phleum pratense*, *Lolium perenne*, *Cynodon dactylon*, and *Secale cereale* were reacted with sera obtained from rabbits immunized with Allergovit, Alutard, Pollinex, Phostal, or BM32 before immunization (*P*) and after 4

monthly intervals (1, 2, 3, and 4). Bound rabbit IgG was detected with iodine 125–labeled antibodies and visualized by means of autoradiography. Molecular weights (in kilodaltons) are indicated on the left margins.

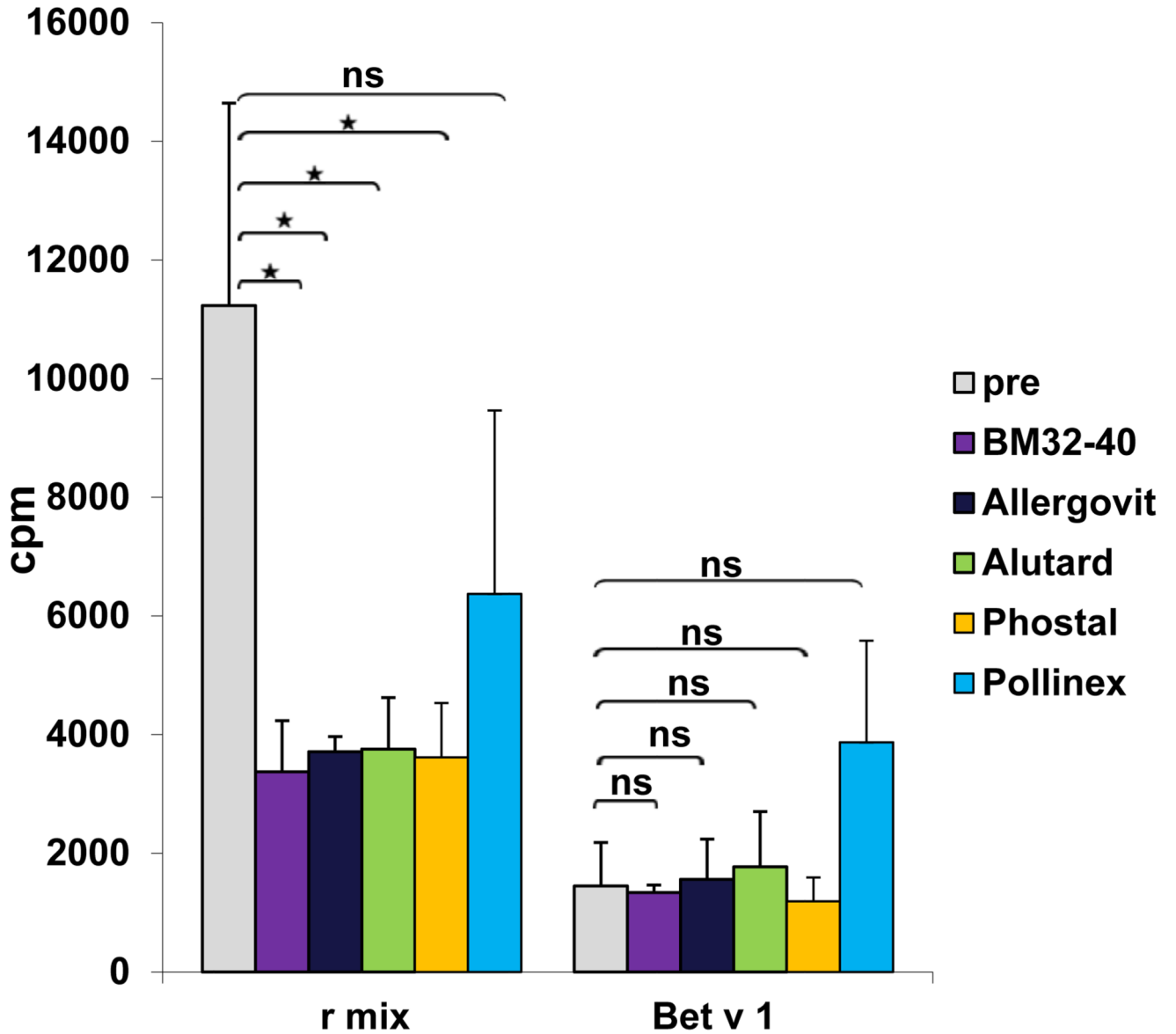


Fig E3.

Rabbit antibodies induced by means of vaccination with BM32 and registered allergen extract–based AIT vaccines inhibit allergen-specific T-cell proliferation. PBMCs from a patient with grass pollen allergy were exposed to a mix of grass pollen allergens or rBet v 1 that had been preincubated with rabbit anti-BM32 IgG antibodies (*violet bars*), anti-Allergovit IgG (*black bars*), anti-Alutard IgG (*green bars*), anti-Phostal IgG (*orange bars*), anti-Pollinex (*blue bars*), or IgG from normal rabbits without immunization (before: *gray*

bars; *x-axis*). Lymphocyte proliferation responses after subtraction of medium background are shown as counts per minute (*y-axis*).

Table E1
Grass pollen allergen-specific IgE levels in sera from patients used for IgE inhibition studies

Patient no.	<i>Phleum</i> species extract (kU _A /L)	Phl p 1 (kU _A /L)	Phl p 2 (kU _A /L)	Phl p 5 (kU _A /L)	Phl p 6 (kU _A /L)
1	669.0	106.7	61.5	125.0	72.0
2	101.0	63.3	ND	81.3	ND
3	132.0	64.4	31.8	64.0	58.3
4	194.0	77.6	38.7	96.9	94.1
5	94.0	95.1	19.0	49.4	37.5
6	83.0	31.0	14.5	50.7	31.2
7	48.0	22.3	11.5	31.5	28.0
8	235.0	90.4	14.2	93.2	83.4

ND, Not determined.

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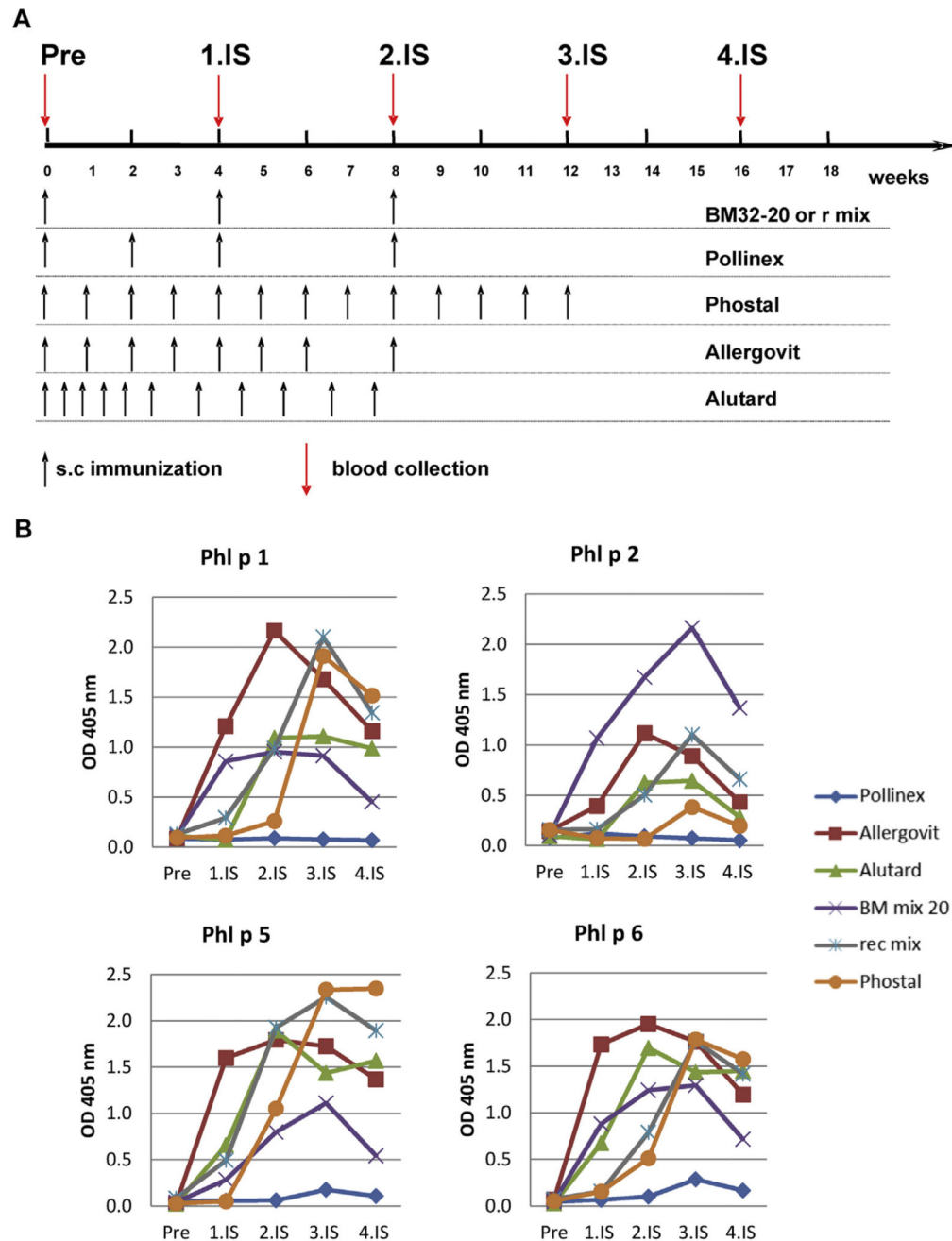


Fig 1. Induction of grass pollen allergen-specific IgG responses in rabbits by means of immunization with BM32, recombinant grass pollen allergens, and natural grass pollen AIT extracts. **A**, Scheme of rabbit immunizations. Rabbits were immunized with 3 monthly injections of BM32 or a mix of recombinant grass pollen allergens. In parallel, rabbits were immunized with registered grass pollen extract-based AIT vaccines (Allergovit, Alutard, Phostal, and Pollinex), as recommended by the manufacturer. Serum samples were collected from all rabbits shortly before the first immunization (*Pre*) and in monthly intervals (*1.I.S.*,

2.IS, 3.IS, and 4.IS). **B**, Comparisons and time courses of allergen-specific IgG responses of rabbits. Shown are mean OD values corresponding to allergen-specific IgG levels (*y-axes*) at different time points (*x-axes*). *s.c.*, Subcutaneous.

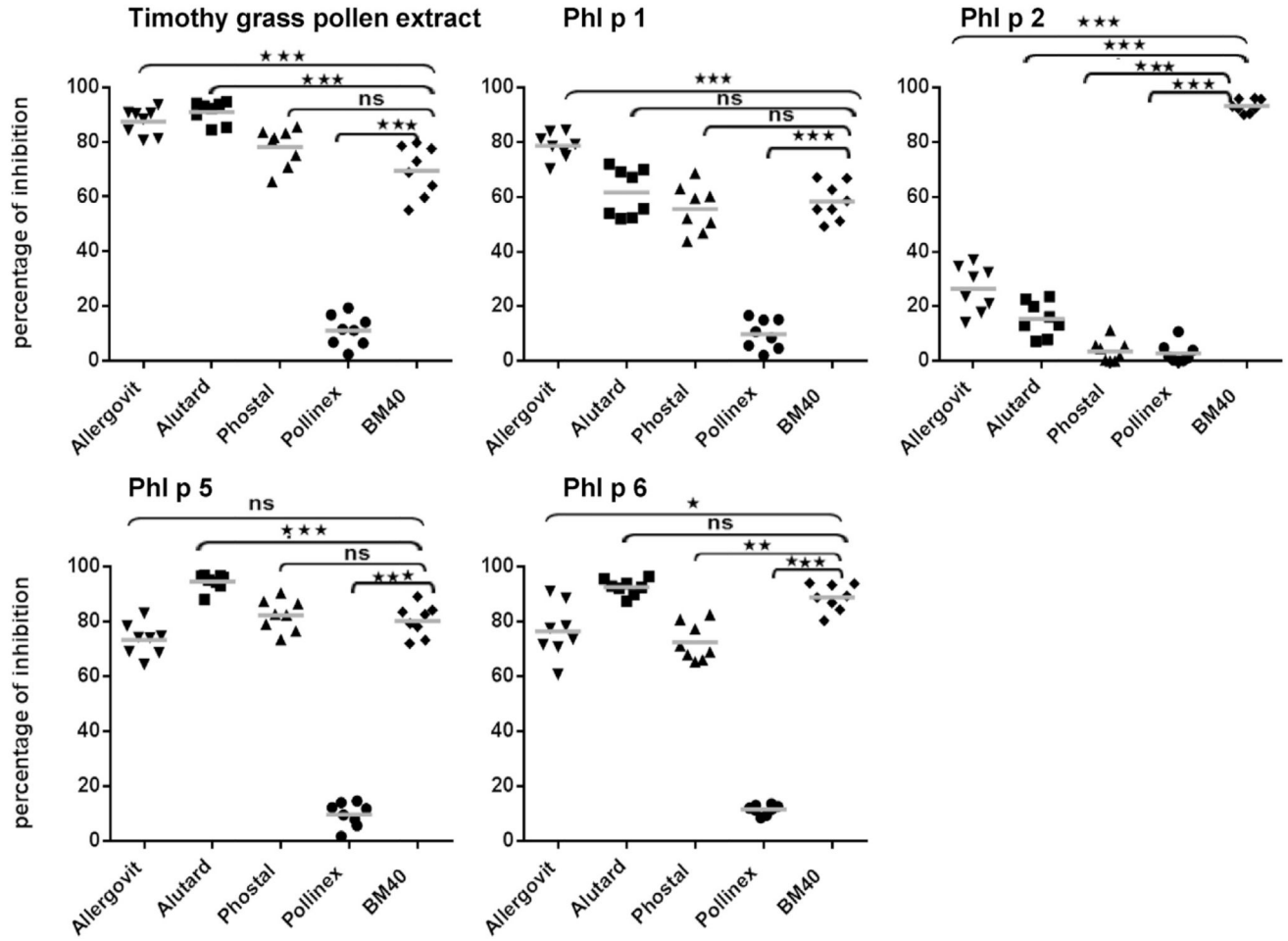


Fig 2. Inhibition of IgE binding in allergic patients to grass pollen allergens with rabbit antibodies induced by vaccination. Shown are the percentages of inhibition of IgE binding (*y-axes*) to timothy grass pollen extract, Phl p 1, Phl p 2, Phl p 5, and Phl p 6 by anti-Allergovit, anti-Alutard, anti-Phostal, anti-Pollinex, or anti-BM32 rabbit antibodies (*x-axes*). Significant differences between BM- and extract-induced antibodies regarding inhibition of IgE binding are indicated. * $P < .05$, ** $P < .01$, and *** $P < .001$. *ns*, Nonsignificant.