

Molecular mimicry between cockroach and helminth glutathione S-transferases promotes cross-reactivity and cross-sensitization

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Background: The extensive similarities between helminth proteins and allergens are thought to contribute to helminth-driven allergic sensitization.

Objective: The objective of this study was to investigate the cross-reactivity between a major glutathione-S transferase allergen of cockroach (Bla g 5) and the glutathione-S transferase of *Wuchereria bancrofti* (WbGST), a major lymphatic filarial pathogen of humans.

Methods: We compared the molecular and structural similarities between Bla g 5 and WbGST by *in silico* analysis and by linear epitope mapping. The levels of IgE, IgG, and IgG₄ antibodies were measured in filarial-infected and filarial-uninfected patients. Mice were infected with *Heligmosomoides bakeri*, and their skin was tested for cross-reactive allergic responses.

Results: These 2 proteins are 30% identical at the amino acid level with remarkable similarity in the N-terminal region and overall structural conservation based on predicted 3-dimensional models. Filarial infection was associated with IgE, IgG, and IgG₄ anti-Bla g 5 antibody production, with a significant correlation between antibodies (irrespective of isotype) to Bla g 5 and WbGST ($P < .0003$). Preincubation of sera from cockroach-allergic subjects with WbGST partially depleted (by 50%-70%) anti-Bla g 5 IgE, IgG, and IgG₄ antibodies. IgE epitope mapping of Bla g 5 revealed that 2 linear N-terminal epitopes are highly conserved in WbGST corresponding to Bla g 5 peptides partially involved in the inhibition of WbGST binding. Finally, mice infected with

H. bakeri developed anti-HbGST IgE and showed immediate-type skin test reactivity to Bla g 5.

Conclusion: These data demonstrate that helminth glutathione-S transferase and the aeroallergen Bla g 5 share epitopes that can induce allergic cross-sensitization. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: GST, cockroach, allergy, hygiene hypothesis, cross-reactivity, filariasis

Epidemiologic and experimental evidence suggests that helminth infections have a general ability to modulate allergic disease¹⁻⁵ through both parasite-induced IL-10 and the expansion of natural regulatory T cells.⁶⁻⁹ Other studies, however, have shown that helminth infections may be associated with increased allergic manifestations,¹⁰⁻¹² perhaps related to the T_H2-dominated environment induced by the parasites¹³ and/or the potential cross-reactivity among allergens and helminth proteins.¹⁴ Indeed, cross-reactivity between helminth tropomyosin and allergenic tropomyosin from mites and cockroaches has been described.¹⁵⁻¹⁷ Although cross-reactivity among tropomyosins has dominated the discussion about helminth-aeroallergen cross-reactivity,¹⁸ several other allergens have orthologues in helminths¹⁹ that may be involved in cross-sensitization.

In the present study, we investigated the cross-reactivity among allergenic glutathione S-transferases (GSTs) and those in pathogenic helminths. GSTs play a variety of physiologic roles including the detoxification of toxins, protection from oxidative stress, and eicosanoid metabolism.²⁰ The cytosolic GSTs can be subdivided into different classes (alpha, mu, pi, sigma, delta, nu, and others), each of which contains 2 basic structural motifs: an N-terminal thioredoxin motif and a C-terminal GST motif that is involved in the formation of the H-site (a hydrophobic pocket that binds substrate). The sequence variability of the C-terminal GST motif confers species specificity to the molecule, making it a target of drug development for allergic diseases, cancer treatment, and parasitic infections²⁰ including vaccines.

Although GSTs are important helminth vaccine candidates,²¹⁻²³ they are known to be strong inducers of IgE. For example, the GST of the cockroach *Blattella germanica* (Bla g 5) is a major cockroach allergen, being an allergen inducing IgE in 30% to 71% of those with cockroach allergies.²⁴⁻²⁶ Moreover, GST is a common allergen of house dust mite (HDM)—with IgE anti-GST being found in up to 96% of HDM-allergic populations.^{27,28}

The present study demonstrated marked similarities at the amino acid (aa) and structural level between filarial (and other

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Abbreviations used

3-D: 3-Dimensional
 aa: Amino acid
 Bla g 5: GST of the cockroach *Blattella germanica*
 DMSO: Dimethyl sulfoxide
 Fil⁺: Filaria-infected
 Fil⁻: Filaria-uninfected
 Fil-A: Fil⁺ and atopic
 GST: Glutathione-S transferase
 HbGST: *Heligmosomoides bakeri* GST-2
 HDM: House dust mite
 Ni-A: Fil⁻ and atopic
 OR: Odds ratio
 WbGST: GST of *Wuchereria bancrofti*

helminth) GST and the major cockroach allergen Bla g 5. We mapped the linear cross-reactive epitopes in humans and showed their involvement in the structural basis for this cross-reactivity. Moreover, we used mice infected with intestinal nematode *Heligmosomoides bakeri* to demonstrate unequivocally that helminth infections induce parasite-specific IgE that sensitizes mice to Bla g 5. These data suggest that helminth infection promotes cross-sensitization to common allergens through molecular mimicry.

METHODS**Patients and sera**

Sera from well-characterized filaria-infected (Fil⁺) individuals were utilized in this study. All patients were seen by the Clinical Parasitology Section of the Laboratory of Parasitic Diseases under protocols approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases and registered (NCT00001230; NCT00001645). The Fil⁺ group in this study was composed of 47 patients with *Loa loa* (n = 37), *Onchocerca volvulus* (n = 6), or *Wuchereria bancrofti* (n = 4). Among the 47, 39 were temporary residents of or travelers to filarial-endemic regions, while 8 were indigenous to these same regions. Sera from 29 filaria-uninfected (Fil⁻; normal) individuals were obtained from the Department of Transfusion Medicine, Clinical Center, National Institutes of Health, under protocols approved by the Clinical Center, National Institutes of Health Institutional Review Board.

All sera were tested for IgE to common allergens by using Phadiatop technology (Phadia, Uppsala, Sweden). Serum samples with levels below 0.35 kUA/L were considered negative and categorized as nonatopic. Sera from Phadiatop-positive subjects were further tested for cockroach-specific IgE by using an Immunocap assay specific for *B germanica* (Phadia). Individuals positive for *B germanica* (levels above 0.35 kUA/L) were considered atopic for cockroach. Based on these data, the 76 subjects were divided into 4 groups on the basis of their cockroach allergy and filarial infection status: (1) Fil⁻ and nonatopic; n = 15 individuals; (2) Fil⁻ and atopic (Ni-A); n = 14; (3) Fil⁺ and nonatopic; n = 11; and (4) Fil⁺ and atopic (Fil-A); n = 36.

Antigens and peptides

cDNA encoding the GSTs of *B germanica* (Bla g 5) or *W bancrofti* (WbGST) were cloned into bacmids. Transformed baculoviruses were used to infect Hi5 cells for the expression of Bla g 5 or WbGST. Cell lysates and supernatants were purified on glutathione columns. The purities of Bla g 5 and WbGST were assessed by using SDS-PAGE. Some experiments utilized recombinant Bla g 5 purchased from Indoor Biotechnologies Inc (Charlottesville, Va). Recombinant Bla g 4 was purchased from Indoor Biotechnologies Inc.

A library of 40 peptides with purities greater than 80% that spanned the entire length of Bla g 5 (see Table E1 in this article's Online Repository at www.jacionline.org) was synthesized by Mimotope (Clayton, Australia).

Each peptide was 15 aa residues in length and overlapped adjoining peptides by 10 residues. Peptide number 31 could not be purified and was not used in the experiments. Peptides were dissolved in HPLC-grade dimethyl sulfoxide (DMSO) (Fisher Scientific, Pittsburgh, Pa) at 10 mg/mL to produce stock solutions and were kept at -40°C until used.

Epitope mapping

An array of 39 overlapping peptides and the full-length Bla g 5 was blotted onto polyvinylidene fluoride membranes by using a 96-well minifold dot blotter (Schleicher & Schuell, Inc, Riviera Beach, Fla). After vacuum aspiration, air drying, and blocking, membranes were incubated with 6 positive and 2 negative sera, depleted of IgG by incubation with protein G beads (GE Biotechnologies, Piscataway, NJ), diluted 1:25 in PBS-milk 5%. Membranes were then incubated with goat anti-human IgE (R&D, Minneapolis, Minn), followed by peroxidase-conjugated rabbit anti-goat IgG or peroxidase-conjugated anti-human IgG (Jackson ImmunoResearch, West Grove, Pa) and developed with enhanced chemiluminescence substrate (Amersham-GE Biotechnologies, Piscataway, NJ) according to the manufacturer's recommendations.

ELISA for Bla g 5 and WbGST

Measurements of Bla g 5, Bla g 4, and WbGST antibodies were performed by using ELISA as previously described.¹⁶ Geometric mean + 3 SD of the antibody levels of Fil⁻ and nonatopic group were used to set cutoff values to identify individuals positive and negative for antibodies to Bla g 5, Bla g 4, and WbGST.

Depletion ELISA

Depletion ELISA was performed as previously described¹⁶ by using Bla g 5, WbGST, or a nonrelated recombinant antigen (OvTropomyosin) to capture specific antibodies and Bla g 5-coated plates to measure the level of depletion.

Inhibition ELISA

Sera samples from infected and noninfected subjects who were IgE and IgG positive for Bla g 5 and WbGST were incubated overnight with 20 µg in 0.1 mL of selected peptides (peptide 2, peptide 7, peptides 12 and 13, peptides 22 and 23, and peptides 27 to 29) dissolved in DMSO (diluted in 1% BSA/PBS from 10 mg/mL stock). Full-length Bla g 5, or WbGST in DMSO at 2% v/v, was used as positive controls, and 2% DMSO was used as negative control. The following day, sera were transferred to plates coated with Bla g 5 or WbGST and developed for IgE antibody reactivity. To increase the sensitivity of the IgE-based assays, these sera were depleted of IgG by protein-G incubation (GE Healthcare, Piscataway, NJ).

Three-dimensional modeling

Three-dimensional (3-D) structural models of Bla g 5 and WbGST were constructed by homology modeling with GST sigma from *Drosophila melanogaster* (DmGST1-1; accession number P41043, PDB 1m0u)²⁹ and GST of *O volvulus* (OvGST2; accession number P46427, PDB 1tu7)³⁰ by using the Protein Model Portal (<http://www.proteinmodelportal.org/>).³¹ The 3-D crystal structure of *H bakeri* GST-2 (PDB 1tw9)³² was used to compare with the models predicted for Bla g 5 and WbGST (see Fig E2 and Table E3 in this article's Online Repository at www.jacionline.org). Molecular visualization of each model and 3-D alignment were performed by using Swiss-pdb viewer (<http://www.expasy.org/spdbv/>).

Sequence alignment and phylogenetic tree

Lasergene MegAlign (DNASTar, Inc, Madison, Wis) was used to compare the sequences of WbGST, Bla g 5, and *H bakeri* GST-2 with themselves or with the protein sequences from other GST allergens of HDMs, cockroaches, and fungus (see Table E4 in this article's Online Repository at www.jacionline.org).

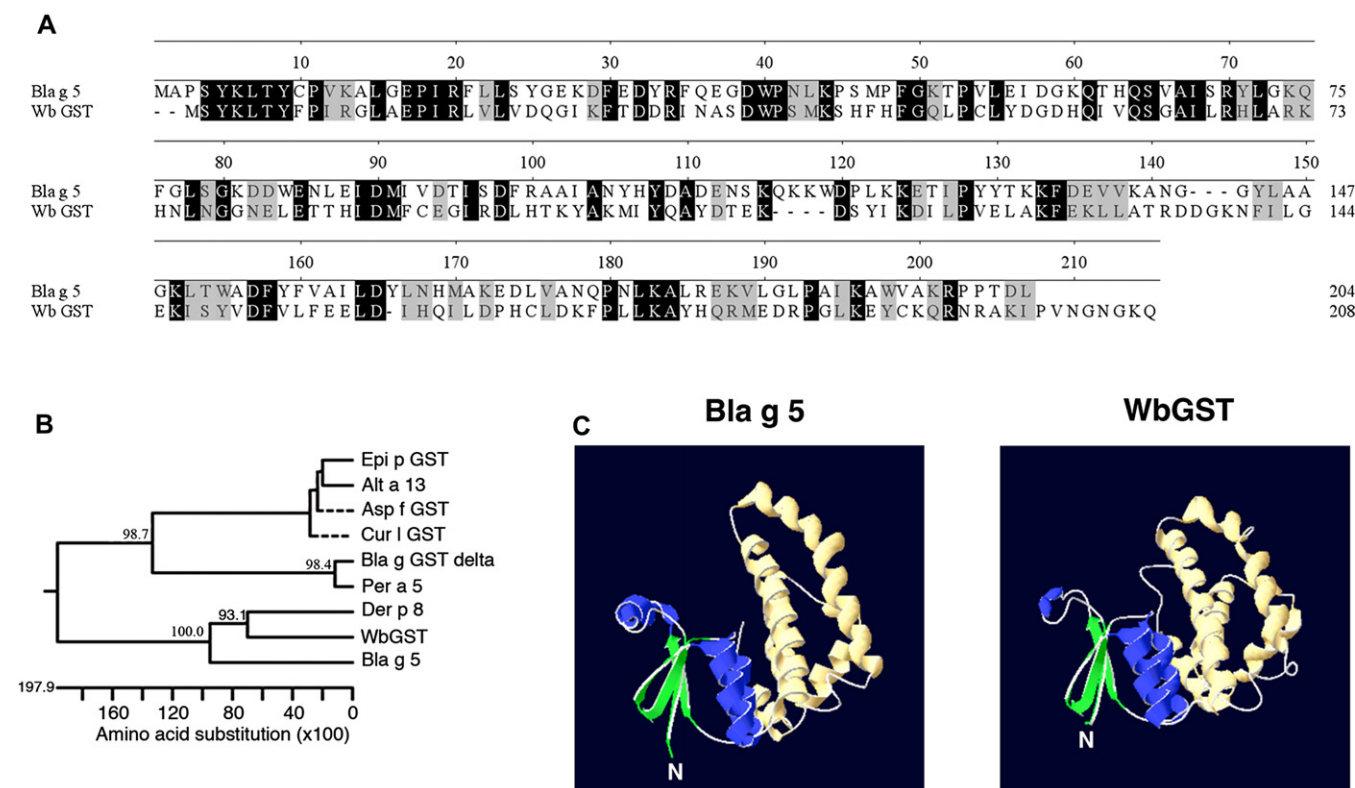


FIG 1. Sequence and structural similarities between Bla g 5 and WbGST. **A**, Alignment of Bla g 5 and WbGST sequences showing identical (shaded in black) and similar (shaded in gray) amino acids. **B**, Phylogenetic tree of allergenic GSTs. **C**, Predicted 3-D structure of Bla g 5 and WbGST color-coding basic structure for comparison.

org). Pairwise or multiple-sequence alignments of the GSTs (Table E4) were performed by using ClustalW (algorithmic Gonet series) and used to construct the phylogenetic tree. Bootstrap values were calculated by using 1000 bootstrap repetition and 111 random seeds.

Mouse infection and skin testing

BALB/c female mice, 6 to 8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, Me), housed at an Association for the Assessment and Accreditation of Laboratory Animal Care–approved facility at the National Institute of Allergy and Infectious Diseases and studied under an animal study proposal approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee. Mice were inoculated *per os* by using a gavage tube with 200 infective third-stage larvae of *H bakeri*. Uninfected control mice (Ni) and mice infected with *H bakeri* for 30 days were skin tested. Mouse ear swelling assays³³ with modifications were performed to evaluate skin sensitivity. Mice were injected with 10 μ g/10 μ L of Bla g 4 (right ear) or Bla g 5 (left ear), and ear thickness was measured with a caliper before and 15, 30, and 60 minutes after allergen injection. Alternatively, mice were injected intravenously with 200 μ L of Evans' blue solution (0.5% w/vol) 3 minutes after intradermal injection of the allergens and euthanized 15 minutes later. Ears were collected, and the Evans' blue dye extracted in formamide overnight at 63°C and quantified in a spectrophotometer at 620 nm.

Statistical analysis

GraphPad Prism v5.0 (GraphPad Software, Inc, San Diego, Calif) was used for all the statistical analyses (1-tailed Fisher exact test, relative risks and odds ratios [OR] with CI, Spearman rank correlation, and Wilcoxon matched-pairs signed rank test).

RESULTS

Bla g 5 and WbGST display related sequence and structure

We investigated the similarities between the cockroach GST allergen (Bla g 5) and a filarial GST from *W bancrofti* (Fig 1). The sequences of Bla g 5 and WbGST were found to be closely related (30% identical, 47% similar) (Fig 1, A). Importantly, the N-terminal region of Bla g 5 and WbGST had a 41% identity over an 80-aa window, which classify them as “likely to cross-react” (World Health Organization criteria³⁴). Phylogenetic analysis that included multiple allergenic GSTs demonstrated that the WbGST clusters closely with Bla g 5 (Fig 1, B). Moreover, the predicted 3-D structures of Bla g 5 and WbGST showed that both GSTs—although from different GST families (sigma and pi)—had well-conserved structures, especially in the N-terminal domain (Fig 1, C).

Antibodies to Bla g 5 cross-react with WbGST

To address the cross-reactivity between filarial GST and Bla g 5, sera from Fil⁺ and Fil[−] individuals were subdivided into cockroach allergic or nonallergic on the basis of specific reactivity to cockroach (*B germanica*) extract, giving 4 groups: (1) Fil[−], non-atopic; (2) Ni-A; (3) Fil⁺ and nonatopic, and (4) Fil-A. The levels of GST-specific IgE, IgG, and IgG₄ were then assessed (Fig 2, A). As can be seen, filarial infection (irrespective of atopic status) was associated with increased levels of IgE, IgG, and IgG₄ antibodies to both WbGST and Bla g 5. When the prevalence of Bla g

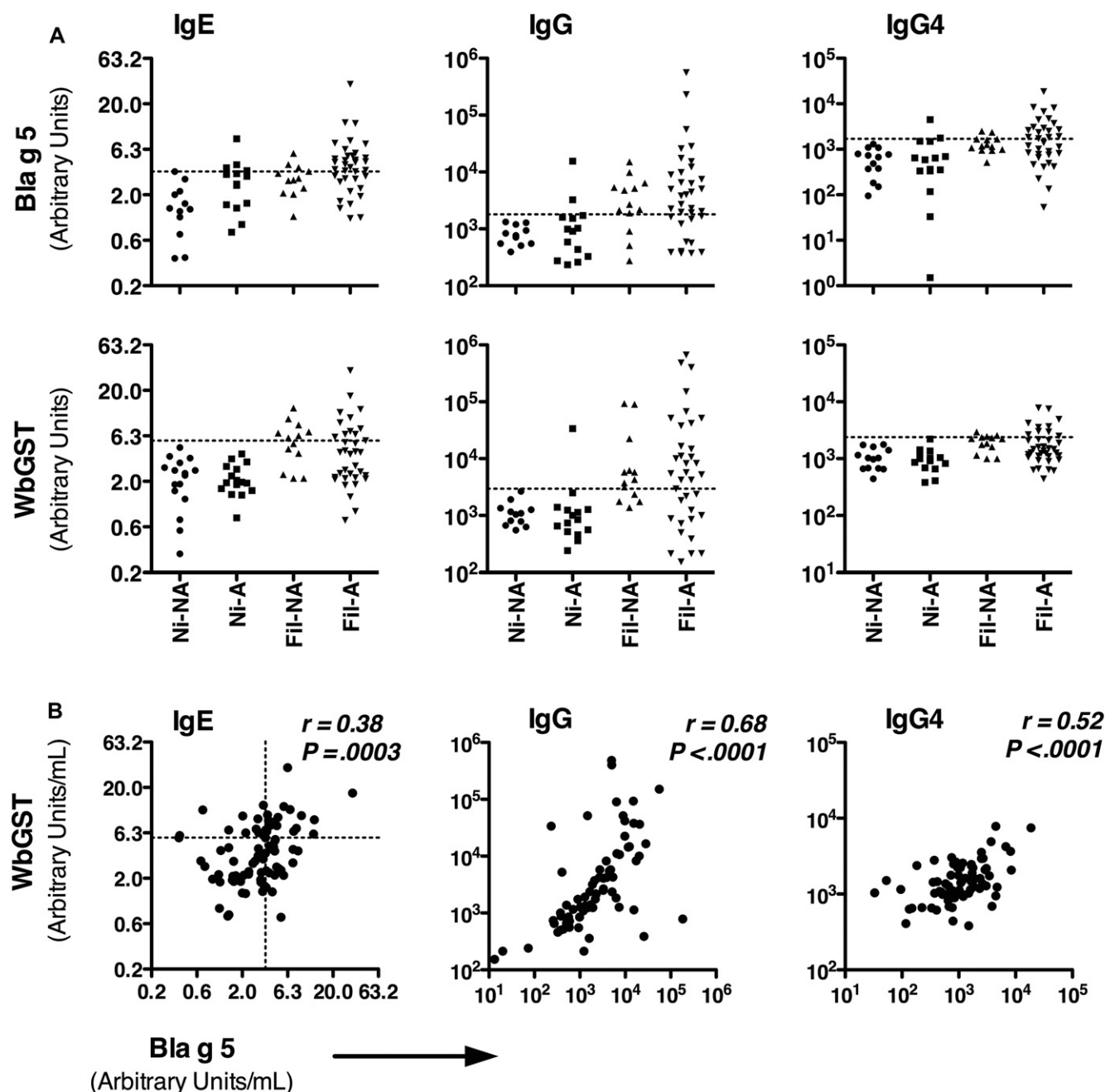


FIG 2. Correlation between WbGST and Bla g 5 antibody levels. **A**, Level of anti-Bla g 5 and anti-WbGST IgE, IgG, and IgG₄ antibodies in noninfected and nonatopic (Ni-NA), Ni-A, filaria-infected and nonatopic (Fil-NA), and Fil-A individuals. Individuals above the dotted lines (representing geometric mean + 3 SD of Ni-NA) were considered positive for anti-Bla g 5 antibodies. **B**, Correlation between anti-WbGST and anti-Bla g 5 IgE, IgG, and IgG₄; each point represents an individual. $n = 76$.

5-specific antibodies was assessed among the atopic individuals (Table I), the presence of filarial infection was associated with increased reactivity to Bla g 5. Indeed, the prevalence of GST-specific IgE was 28.6% (4 of 14) in the Ni-A group compared with 63.9% (23 of 36) in the Fil-A group ($P = .026$). For the anti-GST IgG, the prevalence increased from 14.3% (2 of 14) to 69.4% (25 of 36) ($P = .0005$) and for anti-GST IgG₄, there was an increase in prevalence from 14.3% (2 of 14) to 45% (17 of 36) ($P = .0302$) when Ni-A and Fil-A groups were compared (Table I). Thus, Fil⁺ individuals had significantly increased

likelihoods of being positive for Bla g 5-specific IgE (relative risk of 2.9; OR = 4.4) and IgG (relative risk of 7.0; OR = 13.6) and IgG₄ (OR = 5.3) (Table I). When similar analysis was performed for Bla g 4 (a different cockroach allergen that does not have a parasite orthologue), no significant association between infection status and antibody levels was found. For example, Ni-A and Fil-A showed 8.3% and 31% of anti-Bla g 4 IgE prevalence ($P = .102$), respectively, and 8.3% and 25% ($P = .199$), respectively, for IgG. Furthermore, a positive correlation was found between levels of IgE, IgG, and IgG₄ anti-WbGST

TABLE I. Filarial infection is associated with higher prevalences of anti-Bla g 5 antibodies

	Prevalence (%) [*]		RR (CI)	Odds ratio (CI)	P value [†]
	Ni-A (n = 14)	Fil-A (n = 36)			
IgE	28.6	63.9	2.9 (1.06-8.12)	4.4 (1.15-17.0)	.0262
IgG	14.3	69.4	7.0 (1.75-28.3)	13.6 (2.60-71.5)	.0005
IgG ₄	14.3	47.2	3.6 (0.92-14.7)	5.3 (1.05-27.5)	.0302

RR, Relative risk.

^{*}Prevalence was calculated by using cutoff values as explained in Fig 2.[†]P was calculated by using 1-sided Fisher exact test.

antibodies and the respective levels of anti-Bla g 5 (IgE: $r = 0.38$ and $P = .0003$; IgG: $r = 0.68$ and $P < .0001$; and IgG₄: $r = 0.52$ and $P < .0001$) (Fig 2, B).

To test more specifically the cross-reactivity between the 2 GST proteins, depletion analyses were performed (Fig 3). Preincubation of cockroach GST-positive sera with WbGST was able to deplete 70% of Bla g 5-specific IgE, IgG, and IgG₄, whereas preincubation with a recombinant, structurally unrelated filarial control protein known also to be allergenic (OvTropomyosin)¹⁶ failed to deplete Bla g 5-specific antibodies. Incubation of sera with full-length Bla g 5 or WbGST was able to inhibit the IgE binding to both proteins, but the data suggested that the cross-reactivity was not complete (data not shown).

Involvement of 2 N-terminal linear epitopes in cross-reactivity between Bla g 5 and WbGST

To investigate whether linear epitopes were involved in the molecular basis of Bla g 5/WbGST cross-reactivity, overlapping peptides (15 mers) covering the full length of Bla g 5 (Table E1) were used to map the epitopes involved in IgE and IgG binding (Fig 4) with sera of 4 filaria-infected (numbers 1, 2, 5, and 6) and 2 noninfected (numbers 3 and 4) subjects. As seen, 4 linear regions of Bla g 5 were identified that were reactive to both IgG and IgE: 2 regions in the N-terminal domain (peptide 7, aa residues 31-45 and peptides 12 and 13, aa residues 56-75) and 2 regions in the C-terminal domain (peptides 22 and 23, aa residues 106-125, and peptides 27-29, aa residues 131-155). Three of the regions (peptide 7, peptides 12 and 13, and peptides 22 and 23) corresponded to exposed regions of Bla g 5 that were predicted to be antigenic (Fig 4, B). There was strong reactivity to peptide 7 in 3 of the 6 positive for IgE and 4 of the 6 positive for IgG (Fig 4, A). Four of the 6 had a weak signal to peptides 12 and 13 for IgE, but for IgG there was strong reactivity. The region of the molecule corresponding to peptides 22 and 23, predicted to be highly antigenic (Fig 4, B), had weak but measurable reactivity for IgE (in 3 of 6) and IgG (in 4 of 6) (Fig 4, A). Surprisingly, there was consistent binding of IgE (2 of 6) and IgG (6 of 6) to peptides 27 to 29, a region predicted to be poorly antigenic. We next mapped the 4 antigenic regions to 3-D models of Bla g 5 (see Fig E1 and Table E2 in this article's Online Repository at www.jacionline.org) and compared them to the corresponding regions in the 3-D model of WbGST (Fig E2 and Table E3). Peptide 7 and peptides 12 and 13 were found to be highly conserved between Bla g 5 and WbGST with 40% sequence identity and carbon-alpha root square mean difference of 2.4 Å and 1.7 Å, respectively, at the structural level.

Each of the antigenic peptides was clearly able to partially inhibit the binding of IgE from infected individuals to Bla g 5 (Fig 5). For example, peptide 7, peptides 12 and 13, peptides 22 and 23, and peptides 27 to 29 were able to inhibit the binding of IgE to plate-immobilized Bla g 5 ($P < .04$). In contrast, peptide 2, which was not recognized by any of the subjects tested, could not inhibit binding at all. The fact that the peptides could not completely inhibit IgE binding to Bla g 5 suggests that both nonlinear conformational and linear (possibly conformational) epitopes are involved in the IgE response to this allergen. In the present study, we examined the role played by these linear regions in the cross-reactivity to WbGST. When the peptides were used to inhibit the WbGST ELISA, only peptide 7 and peptides 12 and 13 could inhibit IgE binding, suggesting that the other peptides are not relevant for cross-reactivity. Relevant nonlinear conformational epitopes could not be evaluated in this study.

Conversely, by using sera from filaria-infected patients (see Fig E3 in this article's Online Repository at www.jacionline.org) or Bla g-sensitized mice (intraperitoneal immunizations) (see Fig E4 in this article's Online Repository at www.jacionline.org) in an inhibition ELISA to verify the specificity of the cross-reactivity, we were able to show that peptides from WbGST and GST-2 of *H bakeri*, a rodent helminth (HbGST), corresponding to the Bla g 5 peptide 7 and peptides 12 and 13 could inhibit anti-Bla g 5 IgE equally well (Figs E3 and E4). In addition, Bla g 5 and HbGST peptides could inhibit the WbGST human IgE ELISA (Fig E3, B).

Helminth infection can induce cross-sensitization in mice

To assess whether helminth infection could induce cross-reactive IgE and cause cross-sensitization *in vivo*, we infected BALB/c mice with *H bakeri*, a parasite that contains a GST that is 32% identical (50% similar) to Bla g 5 and contains conserved sequences to the linear epitopes mapped for Bla g 5 (Fig E2 and Table E3), and likely also shows conserved conformational epitopes. More importantly, mice infected with *H bakeri* developed IgE to Bla g 5 (and not to an unrelated cockroach antigen, Bla g 4) (Fig 6, A); further, this IgE mediated immediate-type hypersensitivity responses as measured by intradermally administered antigen (Bla g 5 in one ear or Bla g 4 in the contralateral ear). *H bakeri*-infected mice developed immediate hypersensitivity reactions to Bla g 5 but not to Bla g 4 (Fig 6, B). To confirm this finding, skin-tested animals were injected with Evans' blue dye intravenously 3 to 5 minutes after intradermal injection, and dermal edema was assessed by dye leakage (Fig 6, C). As seen, *H bakeri*-infected mice challenged with Bla g 5 had clear evidence of vascular leakage, whereas Bla g 4 (in the context of *H bakeri* infection) failed to induce an immediate hypersensitivity reaction. This was not due to a failure of Bla g 4 to induce sensitization, as mice sensitized (by intraperitoneal immunizations) to either Bla g 4 or Bla g 5 could mount an immediate hypersensitivity response to the homologous antigen challenge (data not shown). As can also be observed in Fig 6, C, uninfected (Ni) mice reacted to neither Bla g 5 nor Bla g 4.

DISCUSSION

Although the "hygiene hypothesis"⁵ suggests that a decrease in helminth (and/or other) infections may be responsible for the global increase in allergic diseases, there are data to show that

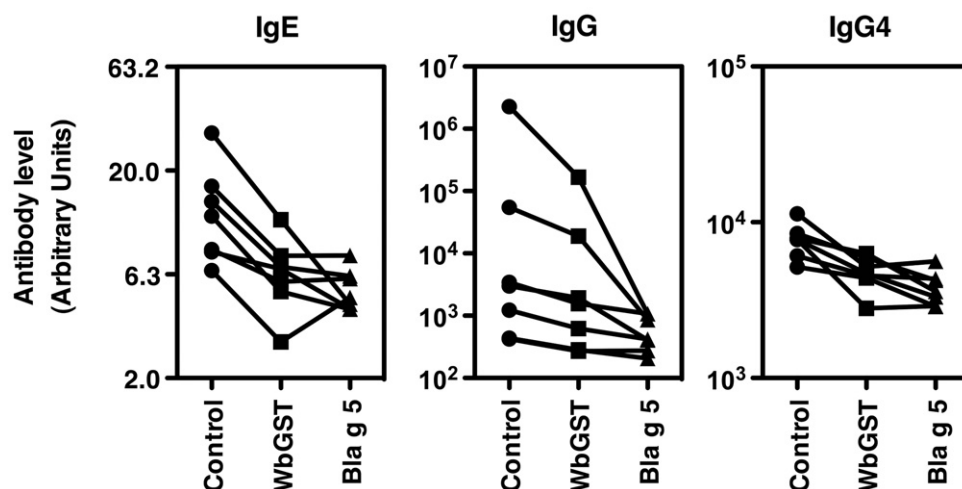


FIG 3. WbGST-specific antibodies cross-react with Bla g 5 because of conserved epitopes. Sera of patients positive for anti-Bla g 5 IgE, IgG, and IgG₄ were incubated in plates coated with a control antigen (OvTro-pomyosin), WbGST, or Bla g 5. Following incubation, sera were transferred to Bla g 5-coated plates and developed for IgE, IgG, and IgG₄. Each dot represents 1 individual.

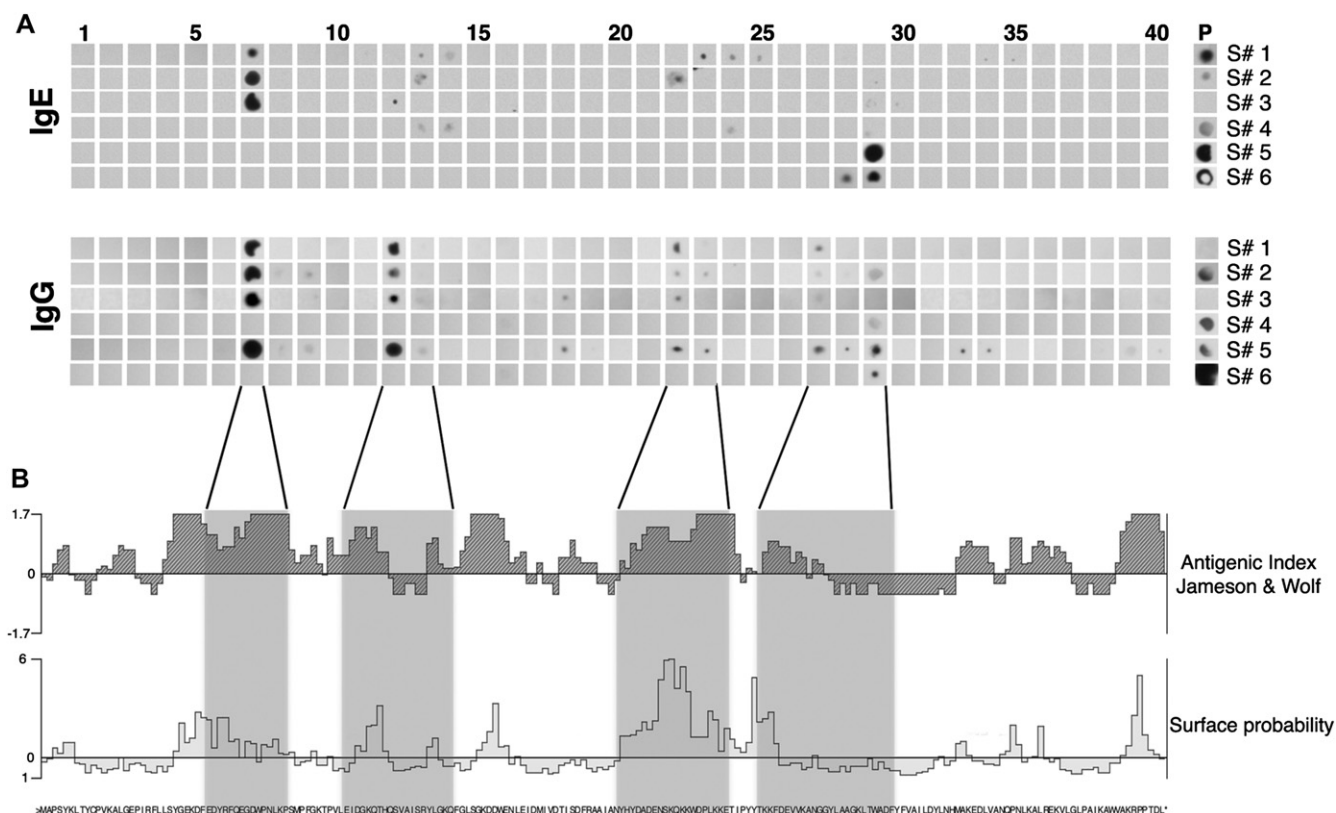


FIG 4. Linear epitope mapping of Bla g 5. **A**, Thirty-nine overlapping peptides spanning the entire sequence of Bla g 5 were used to perform western dot blots for IgE- and IgG-binding epitopes (numbers reflect peptide identity, S stands for serum and P for recombinant Bla g 5). **B**, The results were compared with *in silico* epitope prediction based on the Bla g 5 sequence.

helminth infection can actually increase the risk for atopic disease¹⁰⁻¹² and asthma.³⁵⁻³⁸ One concept that has emerged—in an attempt to understand the allergy-promoting capacity of certain helminth infections—has been the induction of parasite-specific IgE responses that can cross-react with allergens (particularly

aeroallergens), thereby inducing allergic effector responses.^{14-16,19,39}

In regions of the world that are highly endemic for parasitic worm infections, cockroach and HDM are responsible for the overwhelming majority of aeroallergen sensitization.^{10,11,40,41}

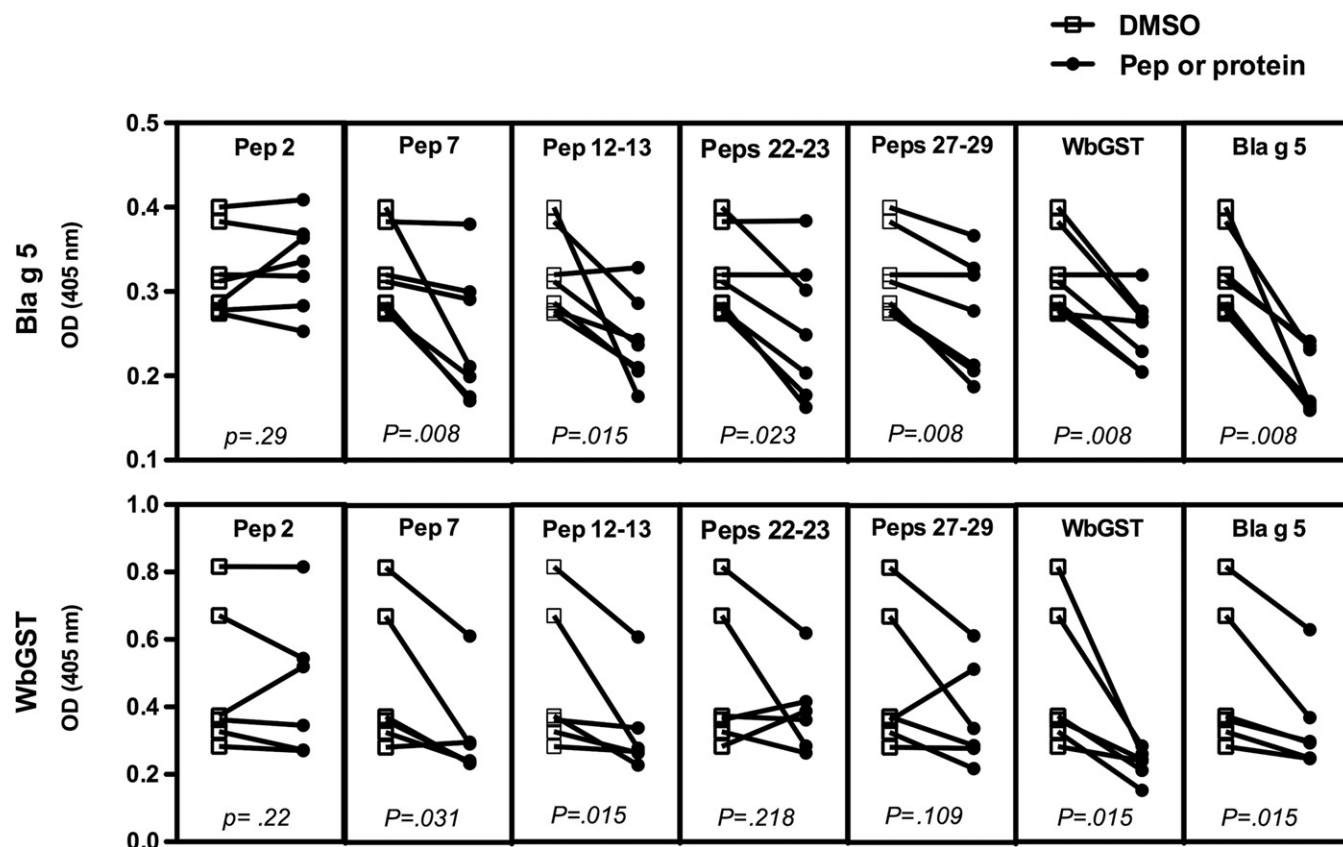


FIG 5. Antigenic peptides can inhibit IgE binding to Bla g 5 and WbGST. Sera from infected patients positive for anti-Bla g 5 and anti-WbGST antibodies were incubated with DMSO (open squares) or specific peptides or recombinant proteins (closed circles) and then transferred to Bla g 5- or WbGST-coated multiwell plates. Optical densities (ODs) are shown for all sera tested. Differences were compared by using the Wilcoxon matched-signed rank test. *Pep*, Peptide.

Among the 10 defined allergens of cockroach in the WHO/OIUIS Allergen Nomenclature Database (www.allergen.org), GSTs (especially Bla g 5) are among the important cockroach allergens.^{24,42} Although the GSTs studied here belong to different families—Bla g 5 is a GST sigma, while WbGST and *H bakeri* GST-2 are GSTs pi and nu—they are evolutionarily related.⁴³ Immunologic cross-reactivity for GSTs of different families that are evolutionarily conserved, although considered to be rare,^{43,44} has been demonstrated for Der p 8 (an HDM GST mu) and Bla g 5 (GST sigma).²⁷ We found by *in silico* analysis of Bla g 5 and WbGST that the N-terminal motif of WbGST displayed 41% identity over an 80-aa residue span, fulfilling the World Health Organization paradigm used to predict cross-reactivity.³⁴ In addition, the 3-D modeling onto which the allergenic linear epitopes were mapped (Fig E2) provided further molecular and structural basis for the cross-reaction seen.

Aeroallergens such as Bla g 5, in contrast with food allergens that tend to have mostly linear epitopes due to proteolytic digestion of the antigen, primarily have conformational epitopes displayed on the surface of the protein. Of note, some linear epitopes can also be conformational if that sequence is surface-exposed,⁴⁵ and, perhaps, this is the case of the linear epitopes identified in this study for Bla g 5. Because the peptides identified as IgE epitopes were unable to completely inhibit IgE binding to Bla g 5 or WbGST, our data suggest that conformational epitopes

are quite important, as suggested by the difference in the potency of ELISA inhibition between the entire protein and the peptides. Nevertheless, our results suggest that the N-terminal portion of Bla g 5, the most conserved region of the molecule, was likely to be the primary region involved in the cross-reactivity to WbGST. These data agree with data from epitope mapping of the bovine filarial parasite *Setaria cervi* GST that has recently shown that the N-terminal end was highly immunogenic.⁴⁶ Because our data suggest only partial cross-reactivity (ie, WbGST could not completely inhibit anti-Bla g 5 antibodies), we conclude that Bla g 5-specific antibodies are composed of both cross-reactive and non-cross-reactive antibodies. The non-cross-reactive antibodies may be directed to a less conserved portion of the molecules or may reflect small differences in the peptide structure or in nonlinear conformational epitopes.

The most important finding in the present study was the demonstration that this induction of cross-reactive, allergen-specific IgE could be recapitulated *in vivo* by using a model of a rodent gastrointestinal nematode in BALB/c mice. We were able to show that this helminth infection induced cross-reactive anti-Bla g 5 IgE that mediated skin sensitivity (immediate hypersensitivity reactions) to Bla g 5 but not to Bla g 4 (a nonorthologous cockroach allergen). The use of Bla g 4 was important to exclude the possibility of helminth-induced polyclonal IgE activation through a T_H2 -skewed immune response that could induce

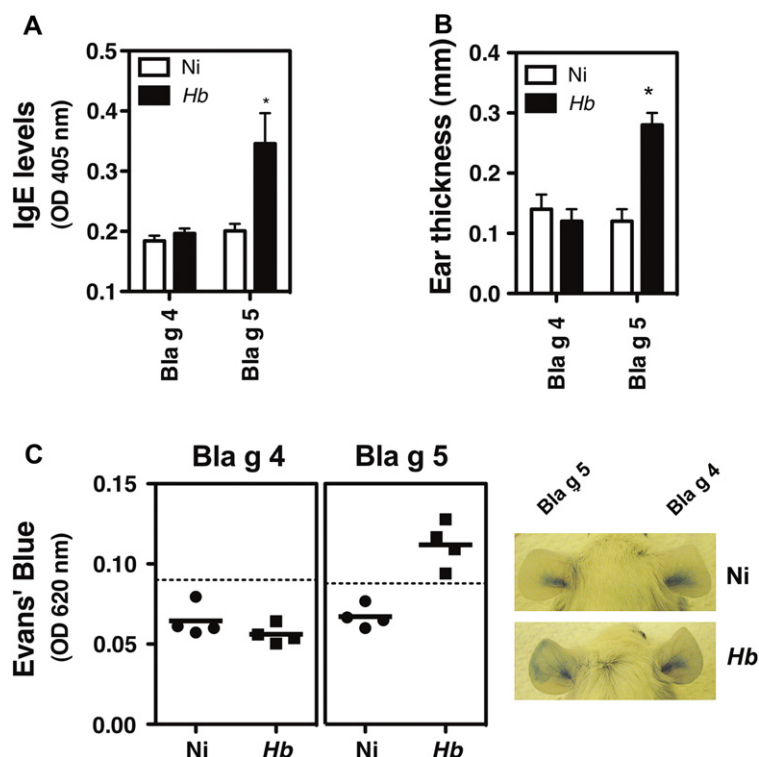


FIG 6. Helminth infection can cause cross-sensitization to Bla g 5. Mice infected with *Heligmosomoides bakeri* developed IgE to Bla g 5 but not to Bla g 4 as measured by ELISA (A) and skin test reactivity by ear thickness (B) or by Evans' blue dye extravasation (C). Data represent 1 of 4 experiments performed (n = 4-5 per group per experiment). Bars (in Fig 6, A and B) represent means $\pm$ SE. In Fig 6, C, each dot represents 1 animal and the dotted lines represent the geometric mean of the Ni group + 3 SD. Hb, *Heligmosomoides bakeri*. * $P < .05$.

indiscriminate allergic sensitization.^{47,48} Our data suggest that cross-sensitization was based primarily on the identity/similarity of the proteins of the parasite to their aeroallergen orthologues.

Cross-sensitization may have important implications for understanding the hygiene hypothesis¹⁶ and to helminth-associated allergy.¹⁴ We show that parasite can induce cross-reactive IgE with possible implications of cross-sensitization *in vivo* that may have significant implications for vaccine development against helminth parasites as suggested by the allergic reactivity (related to the presence of preexisting IgE to the vaccine protein) to a first-generation hookworm vaccine.²² Because GSTs are potential vaccine candidates for *Schistosoma mansoni*,²¹ *Necator americanus*,^{22,49} and *W bancrofti*,²³ the presence of not only pre-existing vaccine-specific IgE but also the potential for cross-reactions to aeroallergen (eg, cockroach) orthologues is of concern.

Together, our data demonstrating induction of cross-reactive allergen-specific antibodies by helminth infection in both humans and experimentally infected mice clearly help to understand the conflicting data that have emerged in the study of the helminth/allergy interface. Not only have we provided a structural basis for the induction of cross-reactive antibodies, but we have also identified concerns that must be addressed in terms of allergy epidemiology, vaccine development,²² and therapeutic approaches to allergic diseases.

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Key messages

- Cockroach and helminth GST cross-react because of remarkable molecular and structural similarities.
- Filarial infection is associated with increased prevalence of cross-reactive anti-Bla g 5 IgE with possible clinical implications.
- Experimental infection with gastrointestinal animal helminth induced cross-reactive allergy as measured by skin test.
- Vaccine development for helminths should be concerned about the potential impact of cross-reactivity with common allergens.

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RESULTS

3-D model of Bla g 5 with the 4 antigenic regions

A homologous GST for Bla g 5 and WbGST with available 3-D structure was searched by using the Protein Model Portal (www.proteinmodelportal.org). The best match available for Bla g 5 was the sigma class GST of *Drosophila melanogaster* (DmGST1-1, accession number P41043, PDB 1m0u), which was 49% identical (69% similarity) and displayed an e-value of 4.0×10^{-57} (Fig E1). The 3-D structure was predicted and the Ramachandran plot analysis revealed that there were 180 (89%) residues in the most favored regions and 19 (9%) residues in additional allowed regions. Although there were 4 (2%) residues in nonallowed regions, they were exposed residues. Two of them were glycine, and the others were also in nonallowed regions in the crystal structure. The quality control of the 3-D Bla g 5 prediction model had a good QMEAN (Qualitative Model Energy Analysis) score (0.77), suggesting good reliability for the model. For WbGST, the best sequence match was the GST of *Onchocerca volvulus* (OvGST2, accession number P46427, PDB 1tu7), which was 79% identical (89% similarity) with an e-value of 2.0×10^{-94} . The Ramachandran plot analysis showed that 189 (91%) amino acids were in the core regions, 12 (6%) residues were in allowed regions, and 7 (3%) residues were in nonallowed regions also present in the crystal structure. The quality control of the WbGST model also showed a robust QMEAN score of 0.77.

Conserved regions for linear epitopes mapped for Bla g 5

Structural localization of Bla g 5 antigenic peptides and its comparison with the correspondent region of WbGST and HbGST. Peptide 7, an important antigenic linear peptide, was predicted to be completely exposed on the protein surface and to consist of a beta sheet followed by an alpha helix, a structure that was retained by the corresponding WbGST and HbGST peptides (Fig E2). Peptides 12 and 13, composed of 2 N-terminal beta sheets and a C-terminal alpha helix (the portion predicted to be poorly immunogenic (Fig 4, B) because of its non-flexible alpha helical structure (Fig E1), are predicted to be hidden. In contrast, peptides 22 and 23 that start with a small turn and then a long alpha helix in Bla g 5 are very different in WbGST and HbGST, especially related to the size of the alpha helix and the positioning of the carbon-alphas (see Table E3). Although Peptides 27 to 29, peptides separated from peptide 23 by just 5 amino acids, are partially conserved, they are formed primarily by a turn. This region and the previous one was found to be weakly antigenic. These results suggested that peptide 7 and peptides 12 and 13, along with relevant conformational epitopes, are likely to be involved in the cross-reactivity between these proteins as demonstrated in the subsequent experiments for Bla g 5 and WbGST and also between Bla g 5 and HbGST as suggested by the *in vitro* and *in vivo* mouse experiments.

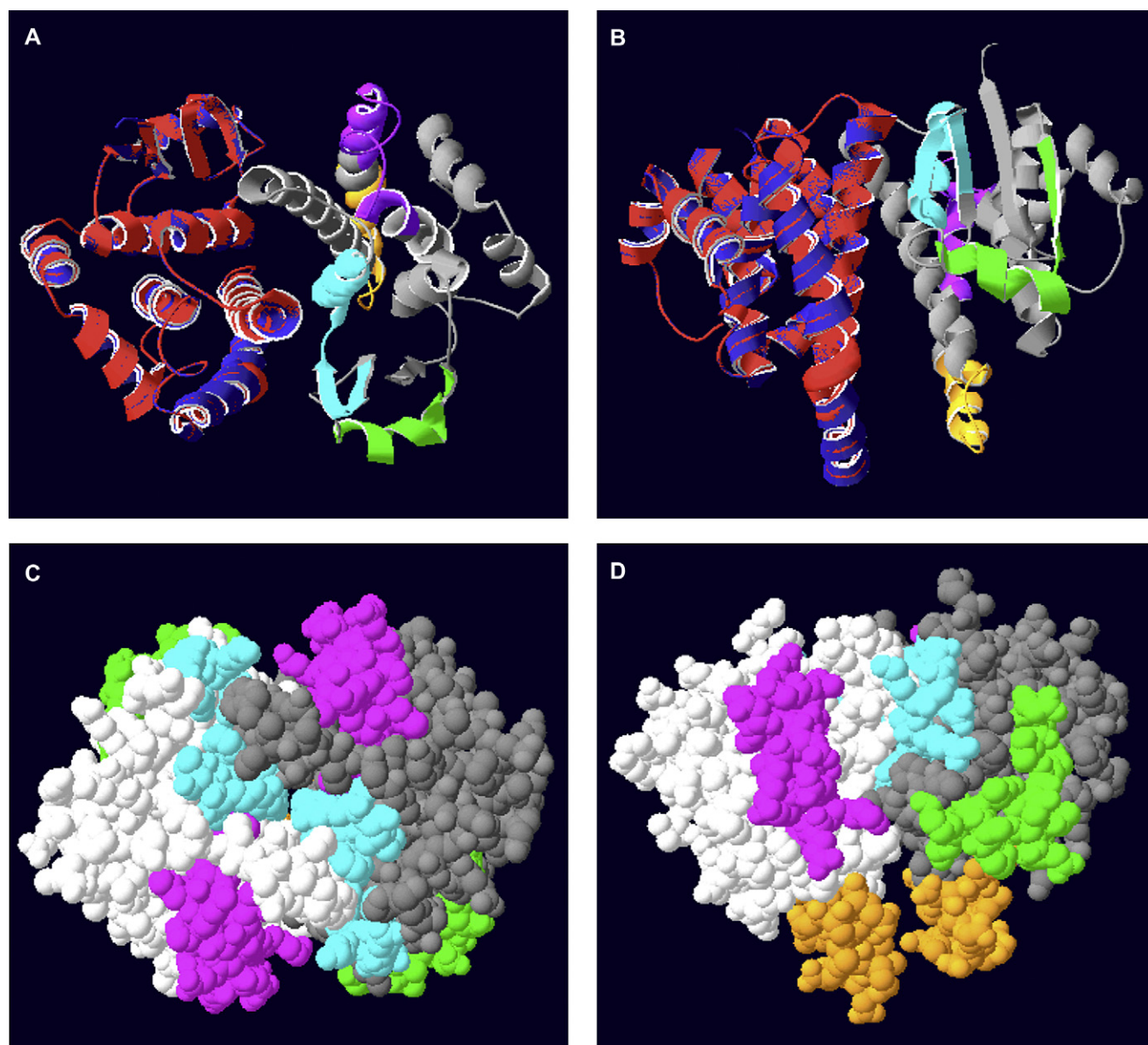


FIG E1. Predicted 3-D structure of Bla g 5 is identical to that of *Drosophila*'s Sigma GST (1m0u). Perfect alignment of predicted monomeric structure of Bla g 5 (**A** and **B**, red) with the crystal model of the dimeric structure of *Drosophila*'s sigma GST where *Drosophila* GST monomers are indicated in blue (left chain) or gray (right chain). Major IgE- and IgG-binding epitopes were marked in cyan (peptide 7), green (peptides 12/13), violet (peptides 22/23), and orange (peptides 27/29). **C** and **D** indicate the spatial distribution of the epitopes in the dimeric *Drosophila* 1m0u structure.

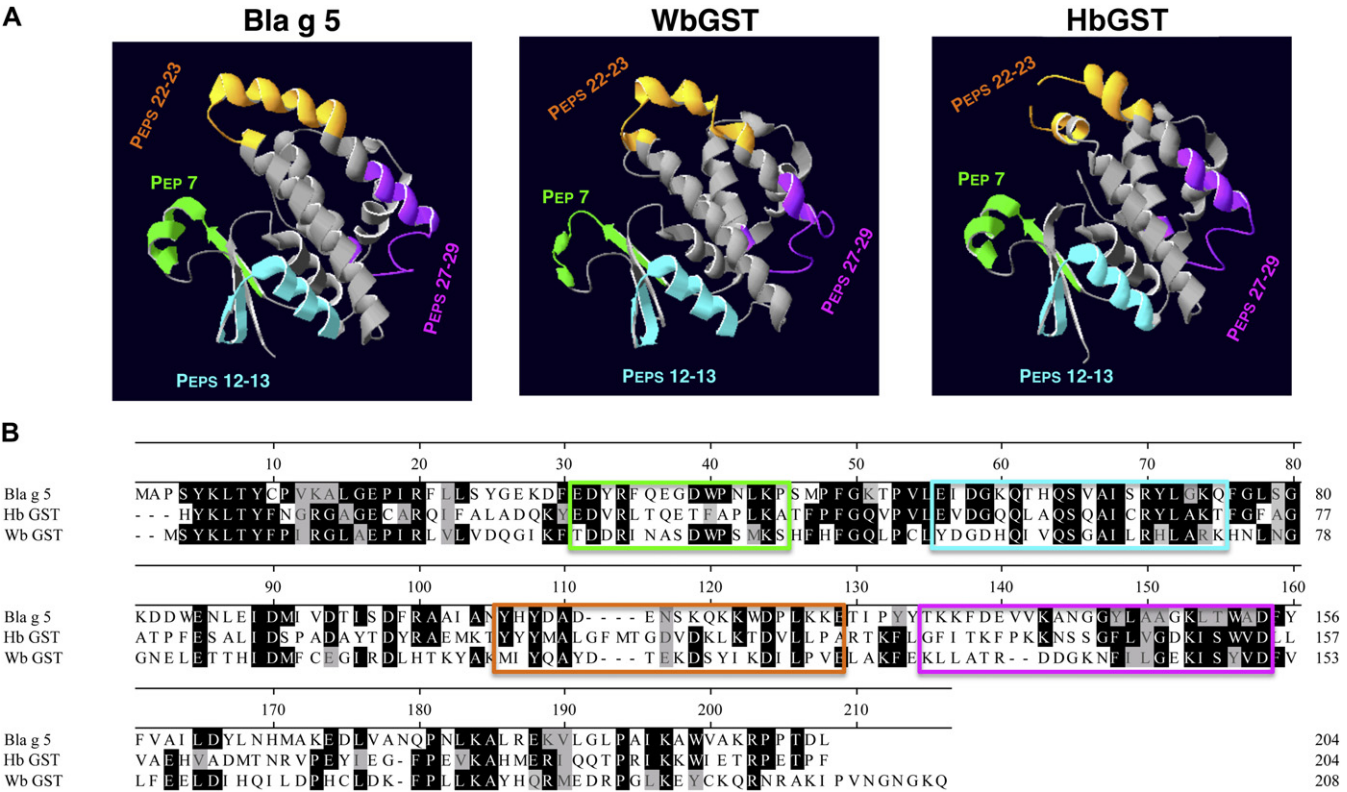


FIG E2. Structural and sequence similarities between Bla g 5, WbGST, and HbGST. **A**, Predicted 3-D structure of Bla g 5 and the correspondent region in WbGST and the crystal structure of HbGST highlighting the linear IgE- and IgG-binding epitopes. **B**, Alignment of the 3 sequences showing identical (shaded in black) and similar (shaded in gray) amino acids. Epitopes are highlighted in the color-coded boxes. *Pep*, Peptide.

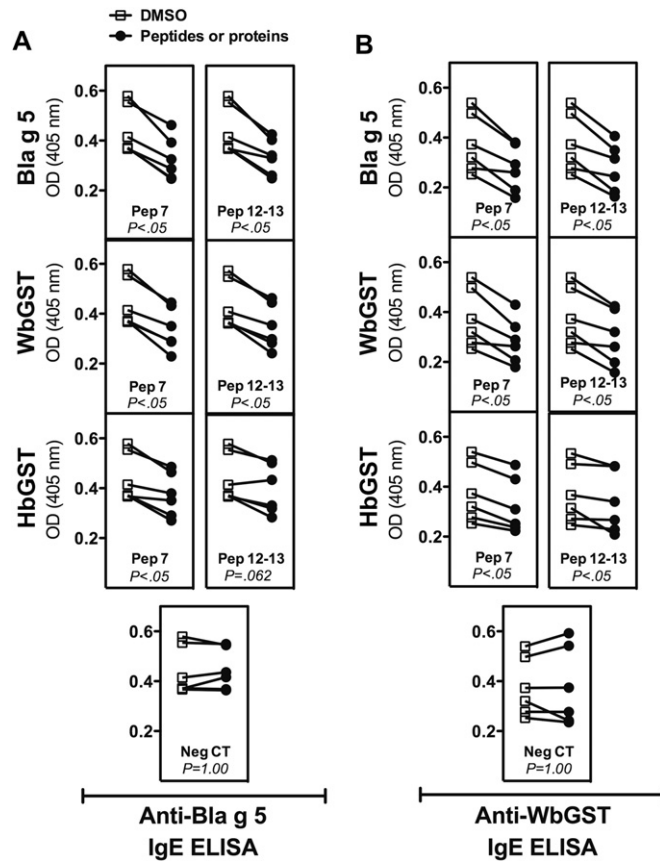


FIG E3. Immunogenic peptides 7 and 12-13 of Bla g 5 and their equivalents in WbGST and HbGST can inhibit (A) Bla g 5 or (B) WbGST-specific IgE in an ELISA. Sera from individuals infected with filarial parasites and positive for anti-Bla g 5 IgE were used to perform the inhibition ELISA as described in the Methods section, except that IgG depletion was not performed for this experiment. Peptide 7 and peptides 12-13 of Bla g 5 and their equivalents in WbGST and HbGST (see Fig E1) were able to inhibit anti-Bla g 5 (Fig E3, A) or anti-WbGST (Fig E3, B) IgE antibody binding. Control (Neg CT) is a non-immunogenic peptide sequence of Bla g 5. *Pep*, Peptide.

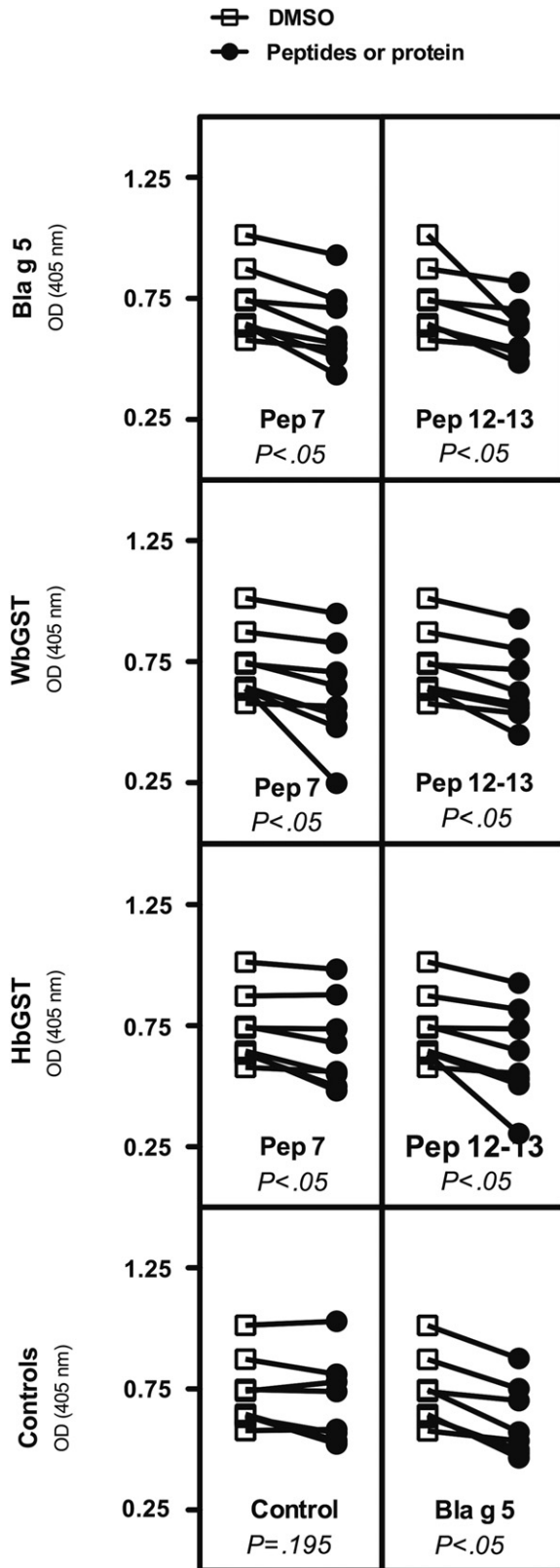


FIG E4. Immunogenic peptides 7 and 12 and 13 of Bla g 5 and their equivalents in WbGST and HbGST can inhibit Bla g 5-specific IgE in ELISA. Sera from BALB/c mice immunized intraperitoneally with Bla g 5 (3 injections of 10 μ g on alternate days without adjuvant) were used to perform the inhibition ELISA as described in the Methods section for human serum, except that IgG depletion was not performed for this

experiment. Peptides 7 and 12-13 of Bla g 5 and their equivalents in WbGST and HbGST (see Fig E1) were able to inhibit anti-Bla g 5 IgE binding in ELISA. Control is a nonimmunogenic peptide sequence of Bla g 5. *Pep*, Peptide.

TABLE E1. List of Bla g 5 linear peptides used in the epitope mapping

	Position	Peptide sequence
Peptide number 1	1-15	MAPSYKLTYPVKAL
Peptide number 2	6-20	KLTYPVKALGEPPIR
Peptide number 3	11-25	PVKALGEPPIRFLLSY
Peptide number 4	16-30	GEPPIRFLLSYGEKDF
Peptide number 5	21-35	FLLSYGEKDFEDYRF
Peptide number 6	26-40	GEKDFEDYRFQEGDW
Peptide number 7	31-45	EDYRFQEGDWPNLKP
Peptide number 8	36-50	QEGDWPNLKPSMPFG
Peptide number 9	41-55	PNLKPSMPFGKTPVL
Peptide number 10	46-60	SMPFGKTPVLEIDGK
Peptide number 11	51-65	KTPVLEIDGKQTHQS
Peptide number 12	56-70	EIDGKQTHQSVASIR
Peptide number 13	61-75	QTHQSVASIRYLQKQ
Peptide number 14	66-80	VAISRYLGKQFGLSG
Peptide number 15	71-85	YLGKQFGLSGKDDWE
Peptide number 16	76-90	FGLSGKDDWENLEID
Peptide number 17	81-95	KDDWENLEIDMIVDT
Peptide number 18	86-100	NLEIDMIVDTISDFR
Peptide number 19	91-105	MIVDTISDFRAAIAN
Peptide number 20	96-110	ISDFRAAIANYHYDA
Peptide number 21	101-115	AAIANYHYDADENSK
Peptide number 22	106-120	YHYDADENSKQKKWD
Peptide number 23	111-125	DENSKQKKWDPLKKE
Peptide number 24	116-130	QKKWDPLKKETIPYY
Peptide number 25	121-135	PLKKETIPYYTKKFD
Peptide number 26	126-140	TIPYYTKKFDEVVKA
Peptide number 27	131-145	TKKFDEVVKANGGYL
Peptide number 28	136-150	EVVKANGGYLAAGKL
Peptide number 29	141-155	NGGYLAAGKLTWADF
Peptide number 30	146-160	AAGKLTWADFYFVAI
Peptide number 31*	151-165	TWADFYFVAILDYLN*
Peptide number 32	156-170	YFVAILDYLNHMAKE
Peptide number 33	161-175	LDYLNHMAKEDLVAN
Peptide number 34	166-180	HMAKEDLVANQPNLK
Peptide number 35	171-185	DLVANQPNLKALREK
Peptide number 36	176-190	QPNLKALREKVLGLP
Peptide number 37	181-195	ALREKVLGLPAIKAW
Peptide number 38	186-200	VLGLPAIKAWVAKRP
Peptide number 39	191-204	AIKAWVAKRPPTDL
Peptide number 40	190-204	PAIKAWVAKRPPTDL

*The peptide could not be purified.

TABLE E2. Comparison between crystal models and predicted 3-D structures*

	Bla g 5 vs 1m0u			WbGST and 1tu7		
	Bla g 5 sequence	Identity (%)	Sequence size (mers)†	WbGST sequence	Identity (%)	Sequence size (mers)
Full length	Accession number O18598	49	204	Accession number Q86LL8	79	208
Peptide 7	EDYRFQEGDWPNLKP	60	15	TDDRINASDWPSMKS	53	15
Peptides 12/13	EIDGKQTHQSVAISRYLGKQ	50	20	YDGDHQIVQSGAILRHLARK	95	20
Peptides 22/23	YHYDADENSKQKKWDPLKKE	30	20	MIYQAYDTEKDSYIKD	75	16
Peptides 27/29	TKKFDEVVKANGGYLAAGKLTWADF	52	25	LAKFEKLLATRDDGKNFILGEKISYVDF	81	28

*Comparison between full-length sequences and the antigenically relevant peptides between Bla g 5 and the crystal structure of *Drosophila melanogaster* GST (1m0u) and WbGST and the crystal structure of *Onchocerca volvulus* GST (1tu7).

†Sequence size of peptides 22 and 23 and peptides 27 to 29 varies because of gaps when the Bla g 5 sequence is compared with the WbGST sequence.

TABLE E3. Conserved linear epitopes mapped for Bla g 5 in WbGST*

	Bla g 5 vs WbGST		Bla g 5 and HbGST	
	Identity (%)	RMSD [†] (Å)	Identity (%)	RMSD (Å)
Full length	30	2.8	32	2.9
Peptide 7	40	2.4	33	1.9
Peptides 12/13	40	1.7	60	1.7
Peptides 22/23	20	4.8	25	6.1
Peptides 27/29	24	3.3	24	3.9

*Comparison of identity level and RMSD[†] of full sequence and antigenically relevant linear peptides between Bla g 5 and WbGST and between Bla g 5 and HbGST.

[†]RMSD, Root mean square deviations in the positions of the corresponding carbon alpha from Bla g 5 and WbGST or Bla g 5 and *H bakeri* GST-2. RMSDs of less than 3.0 Å indicate relatively conserved structures. RMSDs were calculated by using all carbon alpha pairs available.

TABLE E4. List of different GSTs with their respective accession numbers

Species	Name	Accession number
<i>Blattella germanica</i>	Bla g 5	O18598
<i>Wuchereria bancrofti</i>	WbGST	Q86LL8
<i>Heligmosomoides bakeri</i>	HbGST-2	Q9NJQ6
<i>Dermatophagoides pteronyssinus</i>	Der p 8	P46419
<i>Periplaneta americana</i>	Per a 5	Q1M0Y4
<i>B. germanica</i>	Bla g GST delta	A9QUN5
<i>Alternaria alternata</i>	Alt a 13	Q6R4B4
<i>Aspergillus fumigatus</i>	Asp f GST	B0Y813
<i>Curvularia lunata</i>	Cur l GST	Q45X98
<i>Epicoccum purpurascens</i>	Epi p GST	Q45X97