

Identification and characterization of a novel antigen from the nematode *Nippostrongylus brasiliensis* recognized by specific IgE

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Identification and characterization of IgE-inducing antigens are important for elucidating the mechanisms involved in IgE-mediated immune responses in allergic diseases and parasite infections. While many allergens have been characterized, little is known about parasite antigens inducing specific IgE following infection. In order to identify antigens from the nematode *Nippostrongylus brasiliensis*, we generated an IgE-producing B cell hybridoma from *N. brasiliensis*-infected C57BL/6 mice and constructed a cDNA phage display library from *N. brasiliensis*. We successfully cloned and expressed an *N. brasiliensis* antigen (Nb-Ag1) that showed specific binding to anti-*N. brasiliensis* IgE. Nb-Ag1 localized to the pharynx of adult *N. brasiliensis*, suggesting that Nb-Ag1 is a potential pharyngeal gland antigen. Nb-Ag1-specific IgE could be detected in the serum of *N. brasiliensis*-infected mice, but only for a short time and only following a challenge infection. In contrast, local administration of Nb-Ag1 during primary, secondary and tertiary infections induced Nb-Ag1-specific IgE-mediated active cutaneous anaphylaxis. Therefore, amongst the high amounts of polyclonal total IgE, low levels of parasite-specific IgE responses are induced during primary helminth infections. Here, we show that even such low levels of parasite-specific IgE are sufficient to prime mast cells *in vivo* and mediate degranulation.

Received 1/2/07

Accepted 22/2/07

[DOI 10.1002/eji.200737135]

Key words:

Allergy · Antibodies
· Antigens · B cells
· Helminth



See accompanying commentary: <http://dx.doi.org/10.1002/eji.200737135>

Introduction

Elevated levels of serum IgE are characteristic of chronic allergic diseases including asthma, atopic dermatitis, allergic rhinitis, conjunctivitis, and food allergies. IgE

levels also increase in response to nematode parasite infections and, although IgE may play a role in host defense against a few parasites, such as *Trichinella spiralis* [1, 2] and *Schistosoma mansoni* [3], it is not required for expulsion of other parasites, including *Nippostrongylus brasiliensis* [4]. Infection with *N. brasiliensis* is generally believed to generate a polyclonal non-parasite-specific IgE response. This view is based on results obtained from IgE adsorption

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Abbreviations: ACA: acute cutaneous anaphylaxis ·

FcεRI: high-affinity IgE receptor · Nb-Ag1: *Nippostrongylus brasiliensis* antigen · NES: *N. brasiliensis* excretory-secretory ·

PCA: passive cutaneous anaphylaxis

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experiments [5], potentiation of irrelevant IgE specificities [6], and disproportionately elevated total *versus* antigen-specific IgE [7, 8]. These reports support the view that large increases in total IgE levels due to responses to non-related antigens commonly occur in many nematode infections [9] and that induction of non-specific IgE may even provide a means of escape for the parasite [10].

Although the immune response to *N. brasiliensis* infection has been well characterized [11], little is known about *N. brasiliensis* antigens that induce specific IgE responses, and the role of nematode-specific *versus* non-specific antibodies remains elusive. Many studies have used adult worm homogenates or *N. brasiliensis* excretory-secretory (NES) proteins to measure parasite-specific IgE. While in rats NES protein-specific IgE has been observed after both primary and secondary *N. brasiliensis* infection [12], reactivity to NES proteins in mice has only been reported after re-infection [13]. In addition to these crude protein preparations, distinct enzymes have been purified from NES proteins such as Nippocystatin [14] and acetylcholinesterases [15], restricted sets of surface antigens of *N. brasiliensis* have been analyzed [16], and a keratin-like protein of *N. brasiliensis* (KLP-Nb) has been cloned [17]. Despite these studies, no *N. brasiliensis*-derived antigens have yet been reported that are able to mediate biologically important effector functions, such as degranulation of mast cells through cross-linking of the high-affinity IgE receptor (Fc ϵ RI).

Here, we isolated and characterized an *N. brasiliensis*-derived antigen against which a specific IgE response is mounted after infection, in order to investigate parasite-specific IgE responses. First, we generated a B cell hybridoma clone producing monoclonal *N. brasiliensis*-specific IgE and constructed an *N. brasiliensis* cDNA phage display expression library. This library was screened by affinity selection for an *N. brasiliensis*-derived antigen (Nb-Ag1), which showed specific binding to the monoclonal anti-*N. brasiliensis* IgE. Importantly, IgE specific for Nb-Ag1 could be detected in *N. brasiliensis*-infected mice and Nb-Ag1 induced mast cell degranulation *in vivo* in infected mice. Nb-Ag1 appears to be derived from the pharyngeal glands of the nematode, suggesting that this antigen may be an enzyme involved in digestion. Therefore, to our knowledge, we have successfully cloned and characterized the first *N. brasiliensis*-derived antigen that induces a specific IgE response.

Results

Generation of a monoclonal anti-*N. brasiliensis* IgE

In order to identify characteristics of IgE antibodies produced in response to *N. brasiliensis* infection, our first goal was to generate a B cell hybridoma producing monoclonal *N. brasiliensis*-specific IgE. For this purpose, cells isolated from spleen, mesenteric and mediastinal lymph nodes of *N. brasiliensis*-infected mice were fused to a myeloma cell line. The most appropriate time point to perform the hybridoma cell fusion was determined by monitoring the number of IgE-producing cells over time after *N. brasiliensis* infection (data not shown). An IgE-secreting hybridoma clone was successfully generated by fusion of the myeloma cell line X63Ag8.653 with cells isolated from mediastinal lymph nodes 9 days after *N. brasiliensis* infection. The sequence of the monoclonal IgE produced by this hybridoma was subsequently analyzed and the usage of the heavy-chain V(D)J segments as well as the number of mutations within the framework and the complementarity-determining regions were determined. The hybridoma was subcloned and a total of 48 individual subclones were sequenced. All subclones were identical, confirming that the generated IgE was monoclonal (data not shown). Sequence alignment to the closest germ-line sequence revealed that the monoclonal IgE was composed of the V_H9, DSP 2.3, 2.4 or 2.6, and J_H2 gene elements (Fig. 1). Absence of mutations compared to the closest germ-line sequence indicated that this clone had not undergone somatic hypermutation, suggesting that this IgE-switched B cell had not entered a germinal center reaction. For clarity, this monoclonal IgE will be referred to as anti-Nb mIgE.

Identification of an *N. brasiliensis*-derived antigen from a cDNA library

Anti-Nb mIgE was then used to screen an *N. brasiliensis* cDNA phage display expression library in order to identify parasite-derived antigens that are recognized by anti-Nb mIgE. The cDNA library was derived from adult (L₅) *N. brasiliensis* and expressed using the pJuFo phagemid. In order to screen for phages expressing *N. brasiliensis*-derived B cell antigens with a high binding specificity to anti-Nb mIgE, four rounds of panning were performed using the supernatant of the IgE-producing hybridoma. The amplification of cDNA inserts from panned phages showed a decrease in insert diversity following each round of selection, suggesting that panning with the monoclonal IgE was selective (data not shown). After the third and fourth round of panning, PCR amplification of a dominant cDNA insert

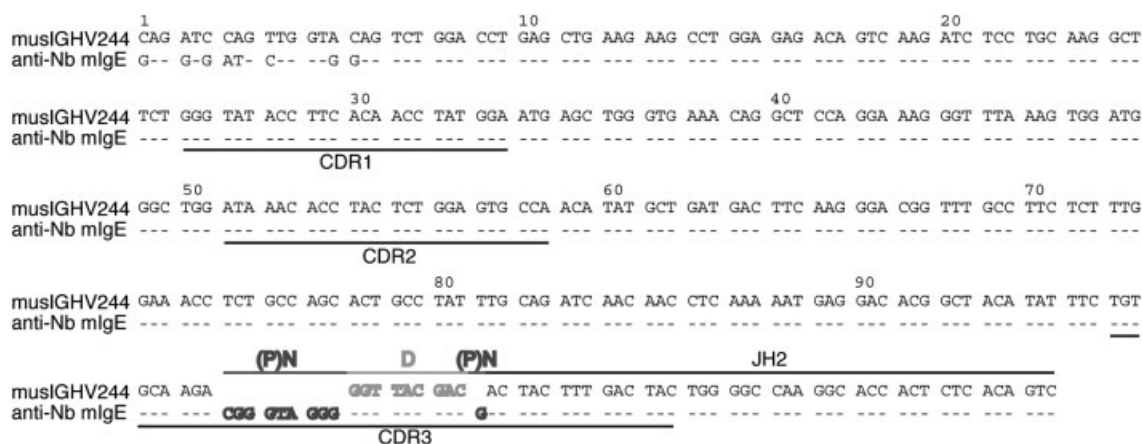


Figure 1. Sequence of anti-Nb mIgE produced by the generated hybridoma. Nucleotide sequences of V, D, and J segments of the anti-Nb mIgE heavy-chain cDNA including putative nucleotide insertions (P)N (dark grey) were aligned to the closest germ-line sequence using the DNAPLOT database (www.dnaplot.de). The V_H segment aligns to the V_H9 germ-line sequence musIGHV244 (using the VBASE2 ID), the D_H segment aligns to the germ-line DSP 2.3, 2.4 or 2.6 element (light grey), whilst the J_H segment aligns to the germ-line J_H2 gene. The first six codons compose the binding site of the degenerative primer mix. Horizontal dashes indicate nucleotide identity.

of approximately 500 bp (data not shown) was revealed. The predominant clone obtained after the fourth round of panning was then sequenced and subcloned into the expression vector pET42a(+), which contains a GST-tag, a His-tag, and a Factor Xa cleavage site upstream of the cloning site. Thus, the 141-amino acid protein, referred to as Nb-Ag1 (Fig. 2A), was expressed as a fusion protein containing N-terminal GST- and His-tags and therefore could be purified through specific binding to a GST-trap column. For simplification, we refer to this GST-His-Nb-Ag1 fusion protein as GST-Nb. As a control, a protein containing the GST- and His-tag without Nb-Ag1 (referred to as GST-Ctrl) was expressed from an unmodified pET42a(+) vector and purified.

After bacterial expression and purification, GST-Nb retained highly specific binding to the anti-Nb mIgE produced by our hybridoma (Fig. 2B). In contrast, anti-Nb mIgE did not show any binding to the GST-Ctrl protein, and IgE of another specificity [anti-2,4,6-trinitrophenyl (TNP)] did not show binding to GST-Nb (Fig. 2B). These results confirmed the successful generation of a monoclonal anti-Nb mIgE and affinity selection of its corresponding specific antigen, Nb-Ag1.

To further characterize Nb-Ag1 and to confirm its size, which was estimated from the amino acid sequence to be 16.6 kDa, we performed Western blot analysis. Our results revealed that GST-Nb was specifically detected by anti-Nb mIgE and had a size of about 48 kDa (Fig. 2C). As the GST-His portion of the fusion protein is known to be 32 kDa, these results confirmed that Nb-Ag1 of approximately 16 kDa was fully expressed in our system. Importantly, the anti-Nb mIgE did not bind to the GST-Ctrl protein or to an unrelated protein (TNP-BSA), and anti-TNP IgE did not bind to GST-Nb. However,

detection with an anti-His (Fig. 2C) or an anti-GST antibody (data not shown) revealed the presence of additional proteins between 25 and 35 kDa in size. These smaller proteins were not detected with the anti-Nb mIgE, suggesting that truncated forms of the fusion

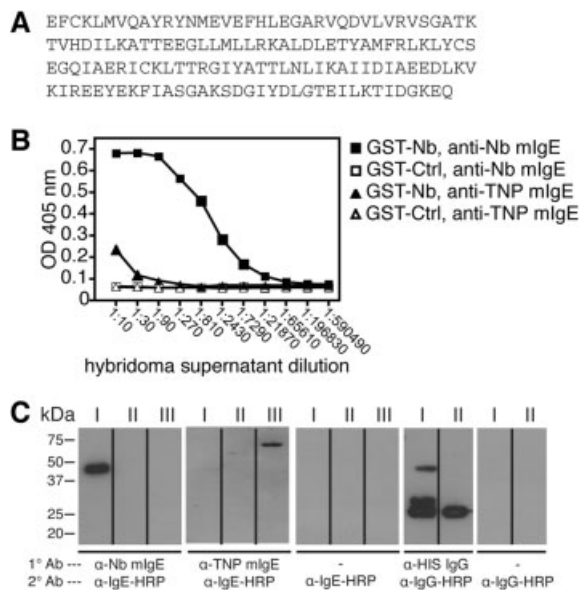


Figure 2. Nb-Ag1 binds specifically to anti-Nb mIgE. (A) Amino acid sequence of the isolated Nb-Ag1 without the GST- and His-tags. (B) Binding of the GST-Nb fusion (filled symbols) or GST-Ctrl (open symbols) proteins to anti-Nb mIgE (squares) or anti-TNP IgE (triangles) was measured by ELISA. (C) Western blot analysis was performed on GST-Nb (I), GST-Ctrl (II), and BSA-TNP (III) and developed with the indicated antibodies. Results are representative of a minimum of three separate experiments.

protein lacking the Nb-Ag1 portion were also expressed, possibly due to premature termination.

Nb-Ag1 induces antigen-specific mast cell degranulation *in vivo*

One of the most important biological functions of IgE is the induction of mast cell and/or basophil degranulation upon cross-linkage of FcεRI. In order to determine whether interaction between the isolated Nb-Ag1 and the generated anti-Nb mIgE could induce mast cell degranulation *in vivo*, we performed a passive cutaneous anaphylaxis (PCA) assay. In this assay, which is performed in rats, intradermal injection of an antibody leads to local mast cell binding *via* surface-expressed FcεRI. After 24 h, antigen is injected intravenously along with Evans blue dye. If the antibody bound to the mast cells is specific to the injected antigen, cross-linkage of IgE receptors will lead to local mast cell degranulation, resulting in increased vasodilation and vascular permeability. Subsequent leakage of serum proteins at the site of mast cell degranulation can be observed by formation of blue spots due to the entry of serum albumin bound to Evans blue dye. Our data showed that cross-linkage of anti-Nb mIgE by the GST-Nb fusion protein was indeed able to induce *in vivo* mast cell degranulation with subsequent local release of active mediators, whilst no such reaction was observed using the GST-Ctrl protein or anti-TNP IgE (Fig. 3). As a positive control, TNP-BSA was also able to mediate cross-linkage of anti-TNP IgE bound to mast cells (Fig. 3). Thus, our recombinantly expressed Nb-Ag1 and the anti-Nb mIgE generated from *N. brasiliensis*-infected mice together mediated the biologically important effector function of mast cell degranulation *in vivo*.

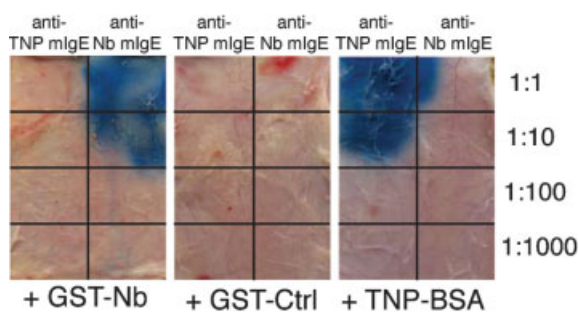


Figure 3. Nb-Ag1 induces mast cell degranulation *in vivo* by cross-linking anti-Nb mIgE bound to mast cells in the skin of rats. PCA was performed in naive Lewis rats by intradermal injection of anti-Nb or anti-TNP mIgE tenfold serially diluted over four steps (as indicated) followed 24 h later by intravenous injection of the GST-Nb fusion, GST-Ctrl, or TNP-BSA proteins in Evans blue dye. Development of blue spots was analyzed 20 min later. Results are representative of three separate experiments.

Induction of Nb-Ag1-specific IgE and IgG₁ antibodies during *N. brasiliensis* infection

Since helminth parasites are known to induce highly polyclonal and non-parasite-specific IgE responses, perhaps in an attempt to subvert protective immune responses, we investigated whether antibodies specific for Nb-Ag1 are induced following infection with *N. brasiliensis*. Following primary *N. brasiliensis* infection, whilst total serum IgE and IgG₁ titers were significantly increased by day 14, neither Nb-Ag1-specific IgE nor IgG₁ were detectable by ELISA (Fig. 4). However, as discussed later, mast cell-bound Nb-Ag1-specific IgE could be detected as early as 7 days after a primary infection with *N. brasiliensis* (Table 1). In contrast, Nb-Ag1-specific IgE could be detected by ELISA following secondary *N. brasiliensis* infection. However, this antigen-specific serum IgE rapidly dropped below the detection limit of our ELISA assay (Fig. 4). Nb-Ag1-specific serum IgG₁ was also detected after secondary *N. brasiliensis* infection and persisted at low levels detectable by ELISA in most of the animals for at least 4 wk (Fig. 4).

Whether the rapid drop of Nb-Ag1-specific IgE below the detection limit of ELISA reflected the very short serum half-life of IgE, although this cannot fully explain our observation since total IgE levels remained elevated for an extended period of time, or whether it reflects an indirect effect due to instability of the Nb-Ag1 antigen, resulting in loss of antigen persistence, remains unclear. *In vitro* we found that recombinant Nb-Ag1 was unstable if it was not stored as a fusion protein (data not shown).

Since specific IgE mediates its major effector function by being cross-linked while bound to the FcεRI on mast cells and not while being present at detectable levels in the serum, we next investigated whether Nb-Ag1-specific IgE is induced and loaded onto mast cells after infection with *N. brasiliensis*.

Parasite-specific IgE and Nb-Ag1 induce mast cell degranulation *in vivo*

The presence of detectable levels of anti-Nb-Ag1 IgE following secondary *N. brasiliensis* infection and the ability of recombinant Nb-Ag1 to mediate mast cell degranulation in a PCA assay (Fig. 3) prompted us to investigate whether mast cells have surface-bound Nb-Ag1-specific IgE following *N. brasiliensis* infection. We therefore performed acute cutaneous anaphylaxis (ACA) assays at various time points after infection. In this assay, purified Nb-Ag1 was injected into the skin of *N. brasiliensis*-infected mice followed immediately by intravenous injection of Evans blue dye. If the IgE produced during the infection was specific to the injected antigen, and sufficient amounts of specific

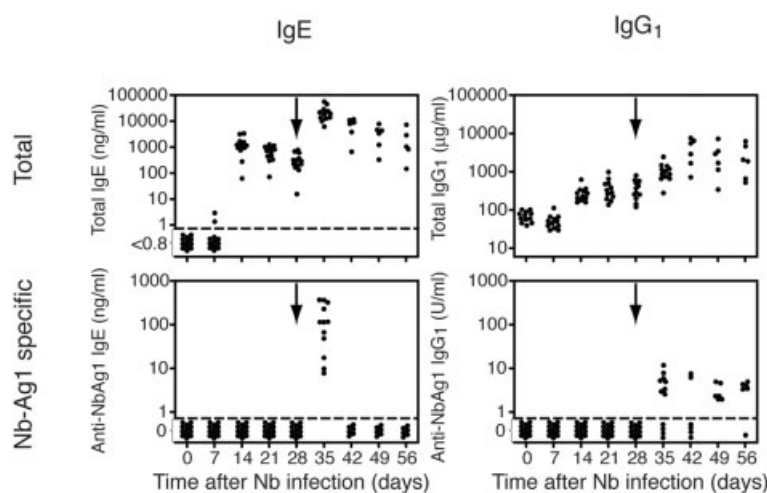


Figure 4. Nb-Ag1-specific IgE and IgG₁ are induced after secondary *N. brasiliensis* infection. C57BL/6 mice were infected with *N. brasiliensis* on day 0 and challenged on day 28 (arrows). Blood was collected at weekly intervals and total and Nb-Ag1-specific IgE and IgG₁ were determined in the serum by ELISA. Nb-Ag1-specific IgG₁ concentrations are indicated in arbitrary units per mL (U/mL). The data points represent individual mice. The dotted lines indicate the lower-limit sensitivities of the ELISA. Results are representative of three separate experiments.

antibody had bound to FcεRI on the surface of skin-resident mast cells, cross-linking of the receptor will induce mast cell degranulation. Although, as mentioned before, Nb-Ag1-specific serum IgE could not be detected by ELISA after primary *N. brasiliensis* infection, we found that GST-Nb antigen mediated specific mast cell degranulation at all time points tested after primary, secondary, and tertiary *N. brasiliensis* infection, whilst injection of an irrelevant antigen (GST-Ctrl) or injection of naive mice did not lead to mast cell degranulation (Fig. 5, Table 1). Therefore, although specific IgE could not be detected by ELISA in the serum following a primary *N. brasiliensis* infection, the ACA revealed that enough Nb-Ag1-specific IgE was induced to bind to mast

cells and 'prime' them for immediate degranulation upon re-encounter with the antigen. This response was IgE mediated as it was absent in IgE^{-/-} mice, and it was not merely the result of elevated total IgE as infection with the enteric helminth *Heligmosomoides polygyrus* did not lead to mast cell degranulation, despite highly elevated levels of total serum IgE (10 508 ± 7413 ng/mL at day 21; Fig. 5, Table 1).

To our knowledge, Nb-Ag1 is the first cloned and characterized parasite antigen able to cause mast cell

Table 1. Nb-Ag1-specific IgE and Nb-Ag1 induce mast cell degranulation *in vivo*^{a)}

Positive ACA/total mice					
Strain	Infection	Day 0	Day 7	Day 14	Day 21
B6	1° Nb	0/6	5/6	5/6	3/3
B6	2° Nb	NA	5/6	4/6	3/5
B6	3° Nb	NA	2/2	2/2	3/3
B6	1° Hp	NA	0/3	0/3	0/3
BALB/c	1° Nb	0/3	3/3	3/3	1/3
IgE ^{-/-}	1° Nb	0/3	0/3	0/3	0/4

^{a)} C57BL/6 (B6), BALB/c, or IgE^{-/-} mice were infected with *N. brasiliensis* (Nb) or *Heligmosomoides polygyrus* (Hp) either once (1°), twice (2°), or three times (3°). At the indicated days post infection, an ACA assay was performed using purified Nb-Ag1. The number of positive ACA mice per total number of mice tested is indicated. NA: not applicable.

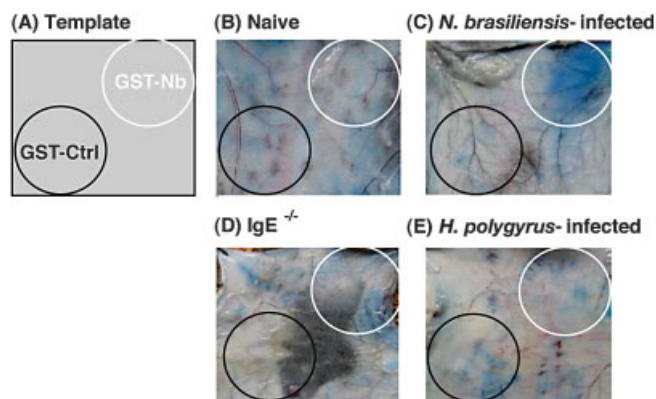


Figure 5. Local administration of Nb-Ag1 induces *in vivo* mast cell degranulation in *N. brasiliensis*-infected mice. GST-Nb (white circles) or GST-Ctrl (black circles) was injected into the skin as indicated in (A). ACA was performed in (B) naive C57BL/6 mice, (C) *N. brasiliensis*-infected C57BL/6 mice 7 days after secondary infection, (D) *N. brasiliensis*-infected IgE^{-/-} mice 14 days after primary infection, or (E) *H. polygyrus*-infected C57BL/6 mice 14 days after primary infection as described in *Materials and methods*. Results are representative of three to six mice per time point.

degranulation *in vivo* after cross-linking specific *N. brasiliensis*-induced IgE bound to mast cells.

Nb-Ag1 is a potential pharyngeal gland antigen of *L*₅ *N. brasiliensis*

In order to identify the anatomical source of Nb-Ag1 in the body of *N. brasiliensis*, we performed immunohistological analysis of the different larval stages (*L*₃, *L*₄, and *L*₅) of the worm. *L*₄ and *L*₅ *N. brasiliensis* were isolated from the lung and gut, respectively, of *N. brasiliensis*-infected *J*_H^{-/-} mice. These mice are deficient in B cells and were used to ensure that no endogenously produced antibodies would be bound to the parasite, which potentially could prevent binding of the detecting antibodies. Fig. 6A shows a bright-field photograph of an *L*₅ worm with various anatomical structures identified. Our anti-Nb mIgE bound specifically to the pharyngeal region of adult *L*₅ stage *N. brasiliensis*, whilst no specific binding was observed using an irrelevant IgE (anti-TNP) (Fig. 6B). We could not detect specific binding of anti-Nb mIgE to *L*₃ or *L*₄ *N. brasiliensis* (data not shown), suggesting that expression of Nb-Ag1 is restricted to the adult stage of the

parasite, or the expression level in other larval stages is below the detection limit of this assay. The pharynx of nematodes is known to function as an efficient pump that maintains high pressure within the body and forces food into the parasite intestine [18]. Notably, two classes of glands secreting digestive enzymes can be found in the second bulb of the pharynx [19]. Considering the immunogenic potency of secreted/excreted *N. brasiliensis* antigens [20, 21], it is likely that Nb-Ag1 derives from such digestive enzymes produced in the pharyngeal glands of *N. brasiliensis*.

Although Nb-Ag1 appeared to be a secreted/excreted protein, preparations of NES from cultured worms, which are traditionally used as a source of *N. brasiliensis* antigens, did not appear to contain Nb-Ag1, as our anti-Nb mIgE did not show specific binding to *L*₃ and *L*₅ NES proteins as determined by Western blot or ELISA (data not shown). Therefore, if Nb-Ag1 is a secreted/excreted protein it must be present in NES protein preparations at very low concentrations. Alternatively, Nb-Ag1 might not be detectable within the NES proteins due to its inherent instability, which leads to rapid degradation of the antigen. Indeed, the low stability of Nb-Ag1 prevented its expression without the GST- and His-tags, as rapid degradation of Nb-Ag1 after cleavage of the GST-His portion was observed (data not shown). In addition, due to the time point (day 9) and origin (mediastinal lymph nodes) from which the anti-Nb mIgE hybridoma was derived one would assume the presence of Nb-Ag1 in early larval stages.

Discussion

We have successfully affinity-selected a novel *N. brasiliensis*-derived antigen, referred to as Nb-Ag1, using a monoclonal anti-Nb IgE to screen a phage display *N. brasiliensis* cDNA expression library. Mast cell degranulation investigated *in vivo* by both PCA and ACA reactions indicated that the interaction of Nb-Ag1 with specific IgE could induce biologically relevant effector functions. This implies that the tertiary structure of Nb-Ag1, although expressed in bacteria, resembles the native form found in adult *N. brasiliensis*. Moreover, beside high levels of non-specific polyclonal IgE, *N. brasiliensis* infection also induced production of parasite-specific IgE at low levels. However, even such low levels were sufficient to bind to the surface of mast cells and mediate degranulation after encounter with their specific antigen (Nb-Ag1). Furthermore, immunohistochemical analysis of adult *N. brasiliensis* revealed that anti-Nb mIgE localized to the pharynx region of adult worms, suggesting that Nb-Ag1 might derive from digestive enzymes produced in the pharyngeal glands. Despite this clue, a BLAST search did not yet reveal a

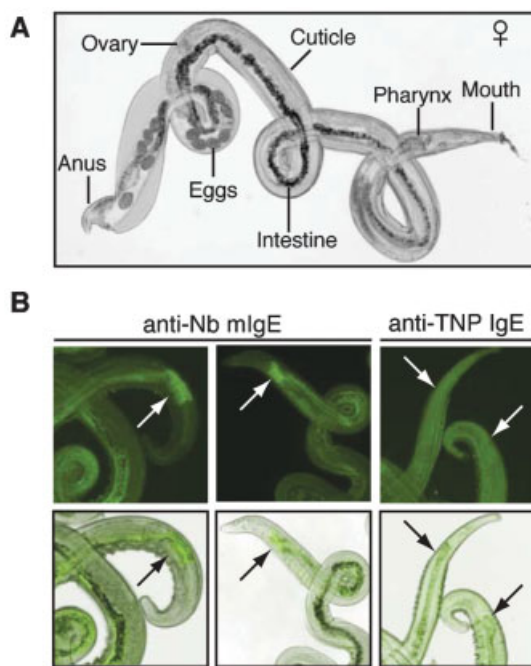


Figure 6. Anti-Nb mIgE binds specifically to the pharyngeal region of adult *N. brasiliensis*. (A) Bright-field photo of a female *L*₅ *N. brasiliensis* worm (isolated from the intestine of *J*_H^{-/-} mice) with various structures indicated. (B) Adult *N. brasiliensis* were incubated with anti-Nb or anti-TNP mIgE followed by anti-IgE-FITC. Arrows indicate the pharyngeal regions of *N. brasiliensis*. The upper panel shows the green fluorescence, the lower panel shows the overlay of FITC staining and the bright-field photo. Results are representative of three separate experiments with a total of about 50 analyzed worms.

known protein with high homology to Nb-Ag1. In addition, analysis of the amino acid sequence did not provide any prediction of characteristic domains within Nb-Ag1. However, as Nb-Ag1 was expressed from a cDNA library, it is likely that it represents only a fragment of a respective *N. brasiliensis*-derived protein.

Nb-Ag1-specific IgE was observed in the serum 7 days after secondary *N. brasiliensis* infection. Surprisingly, although total serum IgE levels remained high, Nb-Ag1-specific IgE decreased rapidly and was no longer detectable 1 wk later. In contrast to serum IgE, the half-life of IgE is significantly prolonged when it is bound to FcεR1 on mast cells [22]. Indeed, by investigating mast cell degranulation in response to locally administered Nb-Ag1, we could detect antigen-specific IgE bound to mast cells at all time points after both primary, secondary, and tertiary *N. brasiliensis* infection. This observation reflects the ability of parasite-specific IgE to mediate immune surveillance within tissues, which is likely to represent the main biological function of IgE.

Primary *N. brasiliensis* infection has been previously shown to induce a polyclonal IgE response, based on the absence of NES protein-specific antibodies in mice [13]. In the present study, the absence of detectable anti-Nb-Ag1 IgE, as determined by ELISA, following primary *N. brasiliensis* infection would support this statement. However, we clearly show two lines of evidence indicating that *N. brasiliensis*-specific IgE antibodies are induced after a primary *N. brasiliensis* infection. Firstly, we succeeded in generating a monoclonal anti-Nb IgE using cells from a mouse that was subjected to only one *N. brasiliensis* infection. Secondly, as early as 7 days post primary infection sufficient anti-Nb-Ag1-specific IgE was induced to mediate a local anaphylactic reaction upon re-encounter with the antigen. Therefore, the absence of NES-specific antibodies in the sera of mice after a primary *N. brasiliensis* infection does not necessarily indicate that the total IgE induced is entirely devoid of *N. brasiliensis* antigen-specific IgE. Nematodes are multi-cellular parasites and are likely to possess many B cell antigens. The primary IgE induced in response to infection may therefore contain many *N. brasiliensis*-specific IgE antibodies, and detection of one or a few of these antibodies amongst the polyclonal response may be difficult. Interestingly, Kanayama *et al.* generated a panel of IgE-secreting hybridomas from *N. brasiliensis*-infected mice, but none of these showed specificity to NES proteins, and therefore it was concluded that none of these IgE clones were specific for *N. brasiliensis* antigens [13]. It would be interesting to screen our *N. brasiliensis* cDNA library with such IgE, as our prediction would be that at least some of the hybridomas might indeed be *N. brasiliensis* specific. Interestingly, if a truly non-specific IgE response were induced by *N. brasiliensis*, one would expect an increase

in IgE of an irrelevant specificity in antibody knock-in mice. However, *N. brasiliensis* infection of quasi-monoclonal mice which express monoclonal antibodies against (4-hydroxy-3-nitrophenyl)acetyl (NP) hapten did not lead to the expected increase in anti-NP IgE [13]. In contrast, secondary rearrangements had occurred such that IgE clones contained VDJ segments different from those found in the parental anti-NP monoclonal antibody. It was concluded that IgE production in *N. brasiliensis*-infected mice is not merely the result of polyclonal activation of B cells, but may involve selection for B cells with specificity.

Sequence analysis of our monoclonal anti-Nb mIgE revealed the absence of somatic hypermutation, suggesting that the B cell clone secreting this antibody had not gone through a germinal center reaction. This may suggest that our cloned *N. brasiliensis* antigen only induces short-lived plasma cells, although binding to mast cells may prolong the half-life of the IgE produced. This notion is further supported by the *in vitro* instability of our antigen such that once the parasite has been cleared, which occurs by 12 days after infection, Nb-Ag1 is likely to disappear as well. This rapid loss of antigen might then lead to the development of only short-lived plasma cells, and therefore antigen-specific IgE would be present in the serum only briefly due to the very short half-life of serum IgE [23]. In contrast, plasma blasts that have been activated in the context of an inflammatory response may be programmed to relocate to inflamed tissues [24], where their survival can be maintained by inflammatory cytokines [25]. Once the inflammation has resolved, such as after expulsion of *N. brasiliensis*, these plasma cells no longer receive survival signals and die.

It is not known whether IgE induction by a primary *N. brasiliensis* infection initially selects many low-affinity B cell clones, or whether the IgE induced is already of high affinity. It would be interesting to evaluate whether IgE undergoes somatic hypermutation (possibly leading to an increase in affinity) after secondary or tertiary *N. brasiliensis* infections.

Investigating the nature of IgE-inducing antigens is crucial for a better understanding of the regulation of IgE-mediated immune responses. In this regard, it is interesting that Nb-Ag1 appears to derive from the pharyngeal glands, which could indicate that it may have an enzymatic function and would therefore be similar to many other IgE-inducing antigens like the dust mite protease Der P1, the cockroach protease Bla g 2, and the bee venom antigen phospholipase A2 [26]. Thus, Nb-Ag1 should facilitate investigations of mechanisms leading to IgE-associated reactions during parasite infections and allergic disorders.

Materials and methods

Mice

C57BL/6 and $J_H^{-/-}$ [27] mice were bred and maintained at the Institute of Laboratory Animal Science, University of Zurich. $IgE^{-/-}$ mice [3] were kindly provided by H. Oettgen and were bred and maintained at the Biologisches Zentrallabor of the University of Zurich. Lewis rats were purchased from Harlan (The Netherlands). All animals were used in accordance with the guidelines of the Kanton Zurich and the Swiss Veterinary Office Animal Protection Laws.

N. brasiliensis infection

N. brasiliensis was maintained by serial passage through Lewis rats. Mice were infected subcutaneously with 550 L_3 larvae.

Generation and sequencing of monoclonal anti-Nb IgE

C57BL/6 mice were infected with *N. brasiliensis*, and mediastinal lymph nodes were removed on days 7 and 9 after infection, while spleen and mesenteric lymph nodes were removed on days 12 and 15. Single-cell suspensions of the organs were fused with the immunoglobulin-non-producing myeloma cell line X63Ag8.653 in a 1 : 1 ratio according to the standard hypoxanthine aminopterin thymidine (HAT) selection method. IgE-producing hybridomas were screened by ELISA. Sequence analysis of anti-Nb mIgE was performed as described [28].

Phage display *N. brasiliensis* cDNA expression library

Adult (L_5) *N. brasiliensis* were collected from the intestines of *N. brasiliensis*-infected C57BL/6 mice on day 7 after infection. RNA was extracted using the Messenger RNA Isolation Kit (Stratagene); cDNA was synthesized, cloned into the Lambda ZAP vector and packaged into XL1-Blue MRF bacteria using the ZAP Express cDNA Synthesis and Gigapack III Gold Cloning Kit (Stratagene) according to the manufacturer's instructions. The size of the primary library was calculated by plating and titrating, and its diversity was determined by amplifying the cDNA inserts in a supernatant aliquot of packaged phages by PCR. The primary library was then amplified and cDNA inserts excised using the ZAP Express cDNA Synthesis and Gigapack III Gold Cloning Kit (Stratagene), and re-cloned into the pJuFo phagemid as described [29].

Selection, expression, and purification of Nb-Ag1

Phages expressing IgE-binding *N. brasiliensis* antigens were affinity-selected using anti-Nb mIgE hybridoma supernatant. Briefly, for each round of panning, ELISA plates were coated with 5 μ g/mL rat anti-mouse IgE (clone 6HD5) and then blocked with 2.5% w/v BSA. Plates were then incubated for 2 h with anti-Nb mIgE followed by incubation with phages (2 h). Bound phages were eluted (0.1 M glycine, pH 2.2) and transformed into XL1-Blue *E. coli*. An aliquot of bacteria was plated, DNA isolated from single colonies and analyzed for diversity by restriction digestion using Eco RI and Xho I. The

remaining bacteria were cultured overnight to amplify the phages. The helper phage VCSM13 (Stratagene; MOI 10) was added to induce secretion of phages, which were collected by PEG-800 precipitation. After four rounds, the pJuFo phagemid encoding the selected *N. brasiliensis* antigen cDNA was isolated from XL1-Blue *E. coli* using the QIAfilter Plasmid Maxi Kit (Qiagen) and the cDNA encoding the *N. brasiliensis* antigen was ligated into the pET42a(+) expression vector (Novagen) and subsequently transformed into BL21-CodonPlus(DE3)-RP *E. coli* (Stratagene). Expression of Nb-Ag1 was induced in the transformed *E. coli* by IPTG. After 3 h, cells were harvested, lysed, and sonicated, and cell membranes were removed by centrifugation. The soluble protein fraction was loaded onto a GST-trap column and a 0–100% gradient of elution buffer containing reduced glutathione was used to elute the GST-tag fusion proteins.

Detection of Nb-Ag1 by ELISA

ELISA plates were coated overnight with GST-Nb fusion protein or GST-Ctrl protein (10 μ g/mL) and blocked with 5% w/v BSA. An initial dilution of 1 : 10 followed by a threefold serial dilution of supernatant collected from anti-Nb or anti-TNP (clone TIB-141) mIgE was added. Bound IgE was detected with HRP-conjugated goat anti-mouse IgE (Bethyl Lab; 1 μ g/mL). ABTS peroxidase solution (0.1 mg/mL ABTS, 0.05% H_2O_2 , and 0.1 M NaH_2PO_4 pH 4.0) was used for the peroxidase-catalyzed color reaction, which was read at 405 nm in a Bio-Rad microplate reader.

Detection of Nb-Ag1 by Western blot

Proteins were separated by SDS-PAGE and transferred to nitrocellulose transfer membranes (Protran; Schleicher & Schuell) using a semi-dry transfer unit (Hoefer SemiPhor). Membranes were blocked with 5% milk/PBS and Nb-Ag1 was detected with anti-Nb or anti-TNP (clone TIB-141) mIgE hybridoma supernatant (1 : 10 dilution) followed by HRP-conjugated goat anti-mouse IgE (Bethyl Lab; 1 μ g/mL). For detection of the GST- or His-tag, HRP-conjugated anti-GST IgG (Amersham; 1 : 5000) or anti-penta-His IgG (Qiagen; 0.1 μ g/mL) followed by HRP-conjugated rabbit anti-mouse IgG (Zymed; 1 : 1000) were used. The membrane was developed using enhanced chemiluminescence (SuperSignal West Pico chemiluminescent substrate; Pierce).

PCA and ACA

Naive Lewis rats, naive or *N. brasiliensis*-infected C57BL/6, BALB/c, and $IgE^{-/-}$ mice, or *H. polygyrus*-infected C57BL/6 mice were anesthetized, a rectangle shaved on their backs and a grid drawn on the skin. For PCA, supernatants of anti-Nb and anti-TNP mIgE (clone TIB-141) hybridoma were tenfold serially diluted over four steps and 50 μ L of each dilution was injected intradermally. After 24 h, 500 μ g antigen (GST-Nb, GST-Ctrl or TNP-BSA) in 0.5% Evans blue dye was injected intravenously. For ACA, 50 μ g antigen (GST-Nb, GST-Ctrl or TNP-BSA) was injected intradermally and 500 μ L of 0.5% Evans blue dye injected immediately intravenously. After 20 min, animals were sacrificed, skin from the back removed and analyzed for mast cell degranulation.

Total, Nb-Ag1-specific and GST-specific serum IgE and IgG₁ measurement

Total IgE or IgG₁ levels were determined as described [28]. For antigen-specific assays, serum antibodies were captured to ELISA plates with rat anti-mouse IgE (clone 6HD5; 5 µg/mL) or goat anti-mouse IgG₁ (Southern Biotechnologies; 1 µg/mL). Plates were blocked with 5% w/v BSA, and serially diluted serum or standards were added and incubated for 2 h. Anti-Nb mIgE hybridoma supernatant was used as standard for Nb-Ag1- and GST-specific IgE; an internal standard consisting of pooled sera of mice immunized twice with GST-Nb was used as standard for Nb-Ag1- and GST-specific IgG₁. Purified GST-Nb or GST-Ctrl was then added (10 µg/mL) and binding was detected with HRP-conjugated anti-GST IgG (Amersham; 1 : 2000). Color reaction was developed and read as described above.

Immunofluorescent staining of *N. brasiliensis*

$J_H^{-/-}$ mice were infected with *N. brasiliensis*. L₄ and L₅ stage *N. brasiliensis* were collected from the lungs on day 2 and intestines on day 6 after *N. brasiliensis* infection, respectively. L₃ stage *N. brasiliensis* were harvested from fecal cultures. Worms were incubated in 1% BSA blocking solution for 30 min followed by incubation with anti-Nb or anti-TNP mIgE hybridoma supernatant for another 30 min. Worms were washed in PBS (2 × 5 min), and incubated in 10 µg/mL anti-IgE-FITC (clone 23G3; Southern Biotech) for 30 min, washed again (2 × 5 min) and then mounted on microscope slides using DakoCytomation fluorescent mounting medium. Fluorescence was monitored on a Zeiss Axiophot microscope with a JVC KYF70 camera, using the Analysis software (Soft Imaging System, Münster, Germany).

Acknowledgements: We thank the staff of the Institute of Laboratory Animal Science of the University of Zurich for animal husbandry and Hans Oettgen for kindly providing the IgE^{-/-} mice. We thank Markus Geuking for helpful comments and for critically reading the manuscript. This work was supported by the Swiss National Science Foundation, grant numbers 3100A0-100779 and 3100A0-100068, the University Hospital Zurich, and a Human Frontier Long-Term Fellowship to K.M.

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