

Cysteine protease of the nematode *Nippostrongylus brasiliensis* preferentially evokes an IgE/IgG1 antibody response in rats

I. KAMATA, M. YAMADA, R. UCHIKAWA, S. MATSUDA & N. ARIZONO *Department of Medical Zoology, Kyoto Prefectural University of Medicine, Kyoto, Japan*

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SUMMARY

Some cysteine proteases such as papain and those of mites and schistosomes have potent allergenic properties. To clarify the allergenicity of nematode cysteine proteases, the enzyme was purified from the intestinal nematode *Nippostrongylus brasiliensis* using cation exchange chromatography and gel filtration chromatography. The purified protease, of 16 kD and pI 8.5, showed maximum enzyme activity at pH 5.5 and substrate preference for Z-Phe-Arg-MCA. The specific inhibitors of cysteine protease leupeptin, iodoacetic acid, and E-64, completely suppressed the activity, indicating that the purified enzyme belongs to the cysteine protease family. Cysteine protease activity was found not only in somatic extract, but also in the excretory-secretory (ES) product of the nematode. When anti-cysteine protease immunoglobulin isotypes were examined in sera from rats infected with *N. brasiliensis*, a high level of IgG1 and a lower level of IgE antibody were detected. Depletion of IgG antibodies from the sera using protein G affinity columns resulted in a marked increase in reactivity of anti-cysteine protease IgE with the antigen, possibly due to the removal of competing IgG antibodies. In contrast to IgE and IgG1, production of anti-cysteine protease IgG2a was negligible. These results indicate that the nematode cysteine protease preferentially evokes an IgE/IgG1 antibody response.

Keywords IgE cysteine protease *Nippostrongylus brasiliensis*

INTRODUCTION

Helminth infections induce marked IgE antibody production [1]. Recent studies have shown that the helminth-induced IgE response is associated with the production of IL-4, and is almost completely suppressed by injection of anti-IL-4 and/or anti-IL-4 receptor antibodies in mice [2–5]. Analysis of CD4⁺ T cell subsets in these mice showed that a large proportion of the helper T cells were Th2 cells, which produce IL-4, IL-5 and IL-10 [6–9]. On the other hand, the response of Th1 cells, which produce interferon-gamma (IFN- γ), was down-regulated [6–9]. Since IgE antibody production is induced by infection with a wide variety of helminths, there may be common properties of helminth antigens that are responsible for triggering such responses.

Recent cDNA analyses of nematode allergens have revealed sequence homology between *Ascaris* major allergen, ABA-1, and surface-associated glycoproteins of filarial parasites *Brugia pahangi*, *B. malayi*, *Wuchereria bancrofti* and *Dirofilaria immitis*

[10–14]. The genes encoding the *Ascaris* and filaria antigens comprise tandem repeats of the coding unit, which gives rise to a very large precursor that is subsequently cleaved to a polypeptide of ≈ 15 kD [10–14]. The filarial antigen was also found to induce a strong IgE response [14].

On the other hand, some cysteine proteases have been shown to be potent allergens. For instance, the plant-derived cysteine protease papain has been reported to induce asthma in workers in meat tenderizer factories [15]. Studies of a potent allergen, *Der p. 1*, purified from house dust mites, showed similarity between the amino acid sequences of *Der p. 1* and known members of the cysteine protease family [16]. That this molecule is a member of this enzyme family was further demonstrated by the finding of intense cysteine protease activity in purified *Der p. 1* preparations as well as in extracts of house dust collected from the homes of mite-induced asthmatic volunteers [17]. Cysteine proteases have been isolated from various species of parasitic helminths [18–23]. Although the potential to act as a preferred target of IgE has not yet been clarified in most helminth-derived cysteine proteases, studies on a schistosome cysteine protease, SMw 32, showed that anti-SMw 32 IgE was detectable after infection in mice [24]. These

Correspondence: Dr N. Arizono, Department of Medical Zoology, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kyoto 602, Japan.

observations raise the possibility that helminth cysteine proteases are one of the common allergens that induce IgE responses after infection.

In the present study, to clarify the possible allergenic nature of nematode cysteine proteases, we purified a cysteine protease from the rodent intestinal nematode *Nippostrongylus brasiliensis*, and examined immunoglobulin isotype responses to this molecule.

MATERIALS AND METHODS

Excretory-secretory product and somatic extract of adult N. brasiliensis at neutral pH

Excretory-secretory (ES) product was prepared by culturing adult *N. brasiliensis* in PBS at pH 7.4 for 24 h at 37°C as described previously [25]. Somatic extract in PBS at pH 7.4 was prepared as described previously [25].

Somatic extract of adult N. brasiliensis at acidic pH

Ten specific pathogen-free, male Sprague-Dawley (SD) rats, 7–10 weeks of age, were injected subcutaneously with 4000 infective-stage larvae of *N. brasiliensis*. Seven days after infection, animals were killed with an overdose of ether, and adult-stage worms of *N. brasiliensis* were recovered from the small intestine. After stringent washing with saline, adult worms were homogenized in 0.2 M citrate buffer (1 g wet weight of worms/2 ml) pH 5.5, with a Polytron (Kinematica AG, Lucerne, Switzerland), and further sonicated for 30 s using an Ultrasonic disruptor (Tomy Seiko Ltd, Tokyo, Japan). The homogenate was centrifuged at 12 000 g for 20 min at 4°C, and the supernatant was recovered.

Enzyme substrates and inhibitors

Synthetic peptides, carbobenzoxy (Z)-Phe-Arg-4-methylcoumaryl-7-amide (MCA), Z-Arg-Arg-MCA, Z-Pyr-Gly-Arg-MCA, Pro-Phe-Arg-MCA, Pyr-Gly-Arg-MCA, Benzoyl-Arg-MCA, Arg-MCA, Glt-Gly-Arg-MCA, Ala-MCA, Succinyl (Suc)-Ala-Pro-Ala-MCA, Suc-Ala-Ala-Pro-Phe-MCA and Suc-Gly-Pro-Leu-Gly-Pro-MCA were purchased from Peptide Institute Inc. (Osaka, Japan). Inhibitors, diisopropyl fluorophosphate (DFP), PMSF, pepstatin A, 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP), leupeptin, and E-64 were purchased from Sigma Chemical Co. (St Louis, MO).

Enzyme assay

Activity was measured by a fluorometric method as described by Barrett [26] with slight modifications. The assay solution (500 µl total volume) contained 10 µl of test sample, 5 mM cysteine and 4 nmol of substrate peptide in 0.1 M phosphate buffer pH 5.5, with or without inhibitors. After incubation at 40°C for 1 h, 1.5 ml stop solution containing 30 mM sodium acetate, 70 mM acetic acid and 100 mM monochloroacetic acid pH 4.3, was added, and aminomethylcoumarin (AMC) released from the peptide was measured with a fluorescence spectrophotometer (F-1200; Hitachi, Tokyo, Japan) at excitation 360 nm and emission 450 nm. The amount of product generated was determined in reference to the fluorometric reading of a standard curve of known amount of AMC. The enzymatic activity was expressed as nmol AMC released per h per ml or mg protein. Protease assays in this study were carried out at 40°C, since the maximal enzyme activity for some cysteine

proteases has been reported to be at this temperature [26,27]. In a preliminary experiment, we also observed that enzyme activity at 40°C was slightly higher than that at 37°C.

Estimation of protein concentration

Protein concentration was determined by the Bradford method using a BioRad protein assay kit (BioRad, Hercules, CA) and bovine serum albumin (BSA) as a standard.

Protease purification

The first step in purification was cation exchange chromatography using a fast performance liquid chromatography (FPLC) Mono S column (Pharmacia LKB, Uppsala, Sweden). The column was equilibrated with 10 mM citrate buffer pH 5.0. The supernatant fraction of the somatic extract at pH 5.0 described above was dialysed against the column equilibration buffer, filtered through a 0.22-µm membrane filter, and applied to the column at a flow rate of 1 ml/min with a linear gradient of 0–1.0 M NaCl dissolved in the same buffer. Fractions (1 ml each) were assayed for protease activity with Z-Phe-Arg-MCA. The enzyme-rich fraction was further subjected to gel filtration chromatography using a Superose 12 column (Pharmacia) equilibrated with 100 mM citrate buffer pH 5.0, at a flow rate of 0.5 ml/min. Fractions (0.5 ml each) were then assayed for protease activity with Z-Phe-Arg-MCA.

Electrophoresis

The purified protease was boiled in a solution containing 2% SDS, 10% glycerol, and 125 mM Tris buffer pH 6.8, with or without 2-mercaptoethanol (2-ME), and electrophoresed in 15% polyacrylamide gel. The molecular weight was estimated in reference to standard molecular markers (Sigma).

Isoelectric focusing was carried out with an EASY-7S electrophoretic system (Funakoshi Co. Ltd, Tokyo, Japan) using 1% agarose plates containing the carrier ampholytes, range pH 3.5–10.6, supplied by the manufacturer. pI calibration marker proteins were purchased from BDH Ltd. (Poole, UK).

Sera

Specific pathogen-free, male SD or Brown Norway (BN/Sea) rats were purchased from Japan Clea Co. (Tokyo, Japan). Eight-week-old animals were injected subcutaneously with 2000 infective-stage larvae as described above, and the animals were periodically bled from the orbital venous plexus. Sera obtained were stored at –80°C until use.

Removal of IgG antibody by protein G affinity column chromatography

A column containing 2 ml of recombinant protein G-Sepharose 4B (Zymed Labs Inc., San Francisco, CA) was equilibrated with 0.02 M phosphate buffer pH 7.0. Two hundred microlitres of test serum (pooled from four to five rats) were applied to the column, and the serum fraction which passed through the gel was collected. As a control, 200 µl of test serum were applied to a 2-ml column containing Sepharose CL-4B (Pharmacia), and the serum fraction passing through the gel was collected. The sera which passed through the protein G and Sepharose CL-4B columns were adjusted to the same protein concentration, and specific IgE, IgG1 and IgG2a antibodies were assayed by ELISA.

Table 1. Protease activity of excretory-secretory (ES) product and somatic extract from *Nippostrongylus brasiliensis*

Substrate	Cysteine	Specific activity (nmol AMC/h per mg)		
		ES	Extract in PBS (pH 7.4)	Extract in citrate buffer (pH 5.0)
Z-Phe-Arg-MCA	+	13.2	12.3	114.4
	-	2.9	3.3	19.0
Z-Arg-Arg-MCA	+	1.8	1.4	4.2
	-	1.4	0.7	0.4

All enzyme assays were performed with or without cysteine (5 mM) in 0.1 M phosphate buffer pH 5.5.

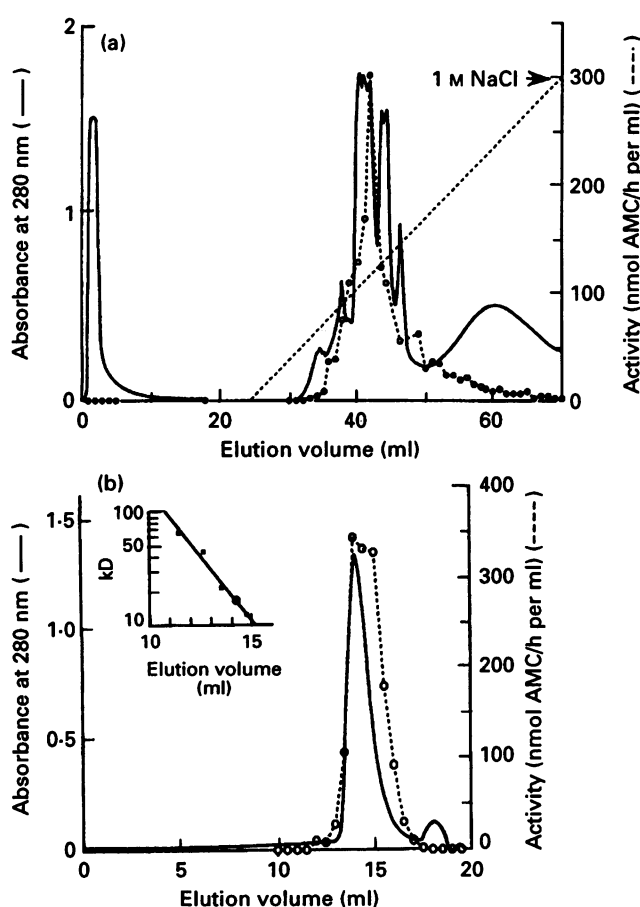


Fig. 1. Purification of cysteine protease from *Nippostrongylus brasiliensis*. (a) Cation exchange chromatogram: a somatic extract solution at pH 5.0 was dialysed against 10 mM citrate buffer pH 5.0, and applied to a Mono S column equilibrated with the same buffer. Bound proteins were eluted with a linear gradient of 0–1.0 M NaCl. (b) Gel filtration chromatogram: active fractions pooled from the Mono S column were applied to a Superose 12 column. In both (a) and (b), the eluates were monitored for protein content, and fractions were assayed for protease activity with Z-Phe-Arg-MCA in the presence of cysteine. Molecular weight markers in gel filtration were bovine serum albumin (mol. wt 66 000), ovalbumin (mol. wt 45 000), soybean trypsin inhibitor (mol. wt 21 500), and horse heart cytochrome C (mol. wt 12 384).

ELISA for antigen-specific IgE, IgG1 and IgG2a

The wells of 96-well immunoplates (Nunc, Roskilde, Denmark) were coated at 4°C overnight with purified cysteine protease (10 µg/ml) diluted with 0.1 M carbonate buffer pH 9.5, and blocked with bovine milk protein (Block Ace; Dainihon Pharmaceutical Co., Osaka, Japan). Appropriately diluted test sera were added and the plates were incubated for 2 h. The plates were then incubated with biotin-conjugated mouse MoAbs against rat IgE, IgG1 or IgG2a (Zymed), then with alkaline phosphatase-labelled streptavidin (Boehringer Mannheim Biochemica, Mannheim, Germany), and were finally reacted with 10 mM 4-nitrophenylphosphate as described previously [28]. After stopping the reaction by adding 3 N NaOH, absorbance at 405 nm was measured with a microplate reader, and the specific absorbance of test samples was calculated by subtracting the absorbance of serum-free wells.

RESULTS

Protease activity in ES product and somatic extracts of adult *N. brasiliensis*

Protease activity of somatic extracts and ES product was measured in a reaction medium pH 5.5, containing Z-Phe-Arg-MCA or Z-Arg-Arg-MCA as substrates. As shown in Table 1, somatic extracts and ES product showed protease activity against Z-Phe-Arg-MCA in the presence of cysteine. The activity was 10-fold higher in the somatic extract at acidic pH than that at pH 7.4 or ES product. Addition of EDTA to the reaction mixture did not reduce the activity. When enzyme activity was measured in reaction medium at pH 7.4 or 8.5, the protease activities in ES product and somatic extracts (pH 7.4 or 5.0) were diminished (data not shown). Enzyme activity in somatic extract (pH 5.0) was abolished by cysteine protease inhibitors (Table 2). These results suggested that the activity was mainly due to cysteine protease.

Purification of protease from adult *N. brasiliensis*

Since protease activity was higher in the extract in 0.2 M citrate buffer pH 5.0 than in PBS pH 7.4 (Table 1), the extract at pH 5.0 was subjected to cation exchange chromatography. A typical elution profile of the supernatant on a Mono S column is shown in Fig. 1a. Peak protease activity was eluted with ≈ 0.45 M NaCl, and the peak fraction resulted in a 6.8-fold

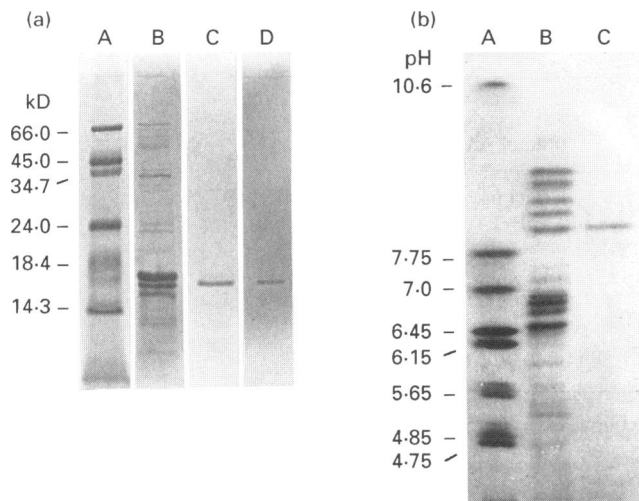


Fig. 2. Electrophoresis of the purified cysteine protease from *Nippostrongylus brasiliensis*. (a) SDS-PAGE. Lane A, molecular weight markers; lane B, somatic extract solution at pH 5.0; lanes C and D, purified cysteine protease with or without 2-mercaptoethanol, respectively. (b) Isoelectric focusing electrophoresis. Lane A, pI markers; lane B, somatic extract solution at pH 5.0; lane C, purified cysteine protease.

increase in specific activity. The active fraction was further subjected to gel filtration chromatography using a Superose 12 column (Fig. 1b). The major cysteine protease activity eluted as a single peak at ≈ 16 kD. Specific activity of the purified protease was 3090 nmol AMC/h per mg, 66.6-fold higher than that of the somatic extract.

The purified protein migrated as a single band on SDS-PAGE, with an estimated molecular weight of 16 kD in both the presence and absence of 2-ME (Fig. 2a). The estimated pI of the protease was 8.5 (Fig. 2b).

Characterization of the purified protease

The substrate specificity of the purified enzyme was examined using 12 kinds of synthetic peptides as described in Materials and Methods. The activity against Z-Phe-Arg-MCA was found to be highest. The enzyme also degraded Pro-Phe-Arg-MCA, Suc-Ala-Pro-Ala-MCA and Z-Arg-Arg-MCA (8.7%, 6.1% and 2.3% of the activity against Z-Phe-Arg-MCA, respectively), but the enzymatic activity to other peptides was negligible. The activity of the purified enzyme to degrade Z-Phe-Arg-MCA was found to be maximum at pH 5.5, and was almost undetectable at $\text{pH} \geq 7.0$. When a variety of protease inhibitors were tested, addition of the cysteine protease inhibitors leupeptin, iodoacetic acid or E-64 abolished the activity, while other inhibitors did not (Table 2). These results indicate that the purified enzyme was a cysteine protease.

Antibody response against the nematode cysteine protease

IgE, IgG1 and IgG2a responses against the nematode cysteine protease were examined in sera from rats infected with *N. brasiliensis*. Using the purified enzyme as antigen, significant levels of anti-cysteine protease IgG1 were detected in rat sera by ELISA (Fig. 3). A low but significant level of anti-cysteine protease IgE was also found to be produced from 2 weeks after infection. On the other hand, anti-cysteine protease IgG2a was

Table 2. Effects of inhibitors on somatic extract (pH 5.0) and purified protease from *Nippostrongylus brasiliensis*

Inhibitor	Per cent of control activity*	
	Somatic extract (pH 5.0)	Purified protease
None	100	100
Without cysteine	17 \pm 0.8	2 \pm 0.1
Metallo		
EDTA (5 mM)	132 \pm 33	123 \pm 2.7
Serine		
DFP (0.1 mM)	91 \pm 5.4	86 \pm 1.6
PMSF (1 mM)	97 \pm 4.4	90 \pm 7.8
Aspartic		
Pepstatin A (2 μ M)	98 \pm 13	106 \pm 3.2
EPNP (1 mM)	80 \pm 16	138 \pm 9.7
Cysteine		
Leupeptin (2 μ M)	4 \pm 0.3	2 \pm 0.8
Iodoacetic acid (1 mM)	1 \pm 0.1	1 \pm 0.8
E-64 (9 μ M)	0 \pm 0.4	0 \pm 0.2

* Samples were preincubated with inhibitors at 40°C for 15 min, and then assayed in 0.1 M phosphate buffer pH 5.5, containing 5 mM cysteine. These results are the mean values \pm s.d. of triplicate assays.

undetectable with the present ELISA method. Since large amounts of anti-cysteine protease IgG1 might have competitively suppressed the binding of IgE to the antigen, IgG-depleted sera were prepared using a protein G column, and applied to an IgE ELISA. As shown in Table 3 and Fig. 3, a marked increase in the reactivity of anti-cysteine protease IgE with the antigen was observed in the IgG-depleted rat sera. When we added back total IgG antibodies eluted from protein G columns to the IgG-depleted sera, the reactivity of IgE antibodies was suppressed dose-dependently (data not shown).

DISCUSSION

Cysteine proteases are found in plants and animals, and some proteases of this family have been reported to be allergenic [15–17,24]. To examine the potential allergenic nature of nematode proteases, a protease was purified from the acid-extracted supernatant of adult *N. brasiliensis*. This protease, of 16 kD, showed an isoelectric point at pI 8.5 and maximum enzyme activity at pH 5.5 in the presence of the SH compound cysteine. This activity was completely suppressed by the cysteine protease inhibitors leupeptin, iodoacetic acid, and E-64, but not significantly by inhibitors of other classes of proteases, serine and aspartic. These characteristics indicate that this nematode protease belongs to the cysteine protease family. The substrate preference of the enzyme for Z-Phe-Arg-MCA is consistent with those of the mammalian cysteine proteases cathepsin B and L [26], and those of *Caenorhabditis elegans* [29], *Schistosoma haematobium* [19], and *Fasciola hepatica* [20].

Healer *et al.* [30] reported that somatic extract of *N. brasiliensis* contains a number of proteases, ranging in size from 51 to ≈ 300 kD. At least some of these enzymes showed properties of metallo-proteases, and were inhibited by a metal chelator 1,10-phenanthroline. The activity of the enzyme

Table 3. Effects of IgG removal on ELISA assay of anti-cysteine protease IgE antibody

	Infection	Antibody titre (OD)	
		Control sera	IgG-depleted sera
IgE	-	0.051	0.079
	+	0.090	0.479
IgG1	-	0.033	0.031
	+	>2.000	0.030
IgG2a	-	0.238	0.052
	+	0.262	0.043

To remove serum IgG, pooled sera obtained at day 0 or 6 weeks after infection were passed through protein G affinity columns. As a control, sera passed through Sepharose 4B were employed. IgE, IgG1 and IgG2a ELISAs were performed using the *Nippostrongylus brasiliensis* cysteine protease (1 µg/well) as an antigen. Data shown are the mean absorbance values of duplicate assays. Note that anti-cysteine protease IgE reactivity of the nematode-infected rat sera increased dramatically after removal of IgG, while anti-cysteine protease IgG1 and IgG2a antibodies were undetectable.

purified in the present study was stimulated in the presence of an inhibitor of metallo-proteases, EDTA, indicating that this enzyme is distinct from metallo-proteases. Such a stimulative effect of EDTA has also been observed for cathepsin B [26], and may be related to the chelation of inhibitory divalent cations. In the study by Healer *et al.* [30], a cysteine protease comparable to that purified in the present study was not detected in *N. brasiliensis* extract. This might be due to a difference in the assay conditions: they examined protease activities at pH 7.6, where cysteine protease activity of *N. brasiliensis* was hardly detectable.

The present study showed that high levels of anti-cysteine protease IgG1 antibody were produced in rats infected with *N. brasiliensis*. This antigenicity suggests that the purified protease is a product of the nematode, and not derived from the host. Although the level of anti-cysteine protease IgE was low in our ELISA system, removal of IgG antibodies from the sera markedly increased the reactivity of specific IgE with the

antigen. When immune IgG antibodies were added back to the IgG-depleted sera, the reactivity of IgE antibodies with the antigen was suppressed. These results suggested that a large amount of anti-cysteine protease IgG1 competitively suppressed the binding of specific IgE. On the other hand, specific binding of IgG2a to the cysteine protease was almost negligible. Thus, immunoglobulin isotype production against the nematode cysteine protease is relatively restricted to IgE/IgG1. Despite the presence of cysteine protease in the somatic extract of adult worms, levels of anti-adult worm extract IgG1 or IgE in the nematode-infected rats were low [31]. ELISA values are known to be significantly affected by the amount of antigen coating the wells of the assay plates. Thus, the lower the content of target antigen in a given amount of crude extract, the lower the ELISA values might become. In this regard, cysteine protease may comprise only a minor fraction of the total extract of adult worms.

Allergenic molecules of *N. brasiliensis* have been partially purified and characterized by several workers. The purified component obtained from homogenized extracts of adult *N. brasiliensis* was a glycoprotein with a molecular weight of 12–17 kD [32] or 12–15 kD [33]. Thus, it is possible that the 16-kD nematode cysteine protease purified in the present study is the same as that reported previously. Cysteine protease activity was found not only in the worm somatic extract but also in the ES product, indicating that the enzyme is secreted from the nematode. Indeed, a 16-kD molecule was detected by immunoblotting analysis of *N. brasiliensis* ES product separated under reducing conditions, although the analysis of ES product separated under non-reducing conditions showed no such 16-kD molecule [25]. The secretory or excretory nature of allergenic cysteine proteases has been reported for *Der p. 1*, which is found in house dust mite excreta [17,34], and in the schistosome SMw 32 which is secreted into the parasite gut [35].

The present study showed that the anti-cysteine protease immunoglobulin response was confined to the IgE/IgG1 isotypes. It is known that *N. brasiliensis* infection induces a dominant Th2 response in mice [36]. A similar response also appears to develop in rats, which show marked increases in IL-4 and IL-5 gene expression after infection (Matsuda *et al.*, submitted). Thus, a question is raised whether, in nematode-infected rats, IgE/IgG1 responses predominate against any

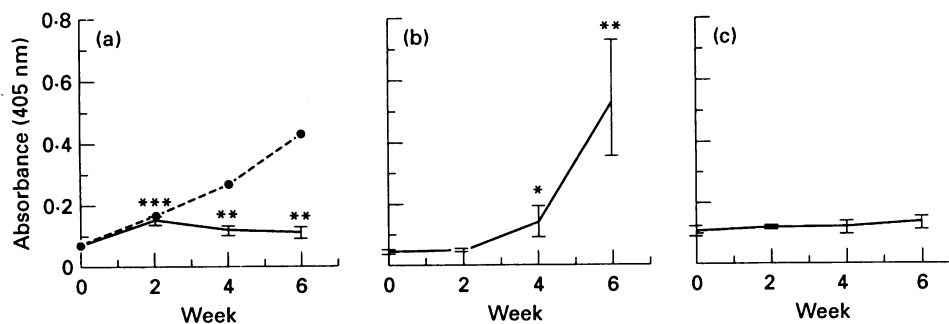


Fig. 3. Anti-cysteine protease IgE (a), IgG1 (b) and IgG2a (c) responses in *Nippostrongylus brasiliensis*-infected BN rats. Sera from uninfected or infected rats, diluted 1:10 for IgE, 1:100 for IgG1 and IgG2a, were applied for ELISA with purified *N. brasiliensis* cysteine protease as antigen. Data shown are means \pm s.d. of four rats. Significantly different from the corresponding values for day 0: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Anti-cysteine protease IgE levels in IgG-depleted sera, prepared by protein G affinity column chromatography, are also shown by a broken line (see also Table 3). When sera from nematode-infected SD rats were examined, similar results were obtained.

worm antigen regardless of the nature of the antigenic molecules. In fact, immunoblotting analyses showed that IgE antibodies from the nematode-infected rat recognized not a single but multiple worm antigens [31]. However, in *N. brasiliensis*-infected rats, not only IgE/IgG1, but also a substantial IgG2a response was generated and the major IgE- and IgG1-binding molecules were identical, while some, but not all, of the major IgG2a-binding molecules were different from the IgE/IgG1-binding molecules, suggesting that IgE/IgG1 responses are preferentially induced by some of the worm antigens but not by others [31]. Similarly, a recombinant antigen-allergen chimaeric protein was reported to induce different immunoglobulin isotypes in mice against the two components: IgG2a against the antigen component and IgE against the allergen component [37]. Furthermore, Soloway *et al.* [38] reported that variants of synthetic peptides differed greatly in their capacity to induce hypersensitivity in mice, and this was associated with the production of IL-4. Taken together, data indicate that cysteine protease might be one of several *N. brasiliensis*-derived allergens which induce dominant IgE/IgG1 responses.

The possibility that the proteolytic activity of cysteine proteases has some function in the induction of IgE antibody response is intriguing. Finkelman & Urban [39] hypothesized that some proteolytic enzymes, which are produced in large quantities by many helminth parasites, are triggers of cytokines that induce a Th2 response. In addition, they reported that injection of papain into the footpad of mice triggered, within 1 h, increases in IL-4, IL-5 and IL-9 mRNA levels, but not those of IFN- γ or IL-2 mRNA [39]. We are currently engaged in studies of immunoglobulin isotype production as well as T cell cytokine responses upon *in vivo* injection of the nematode cysteine protease.

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