IgE and IgG2a antibody responses are induced by different antigen groups of the nematode *Nippostrongylus brasiliensis* in rats

M. YAMADA, M. NAKAZAWA & N. ARIZONO *Department of Medical Zoology, Kyoto Prefectural University of Medicine, Kyoto, Japan*

SUMMARY

The differences were examined between IgE, IgG1 and IgG2a antibody responses against two kinds of nematode antigens in rats infected with *Nippostrongylus brasiliensis*. With ELISA studies, remarkable IgE and IgG1 antibody responses were observed against antigens in excretory/secretory products (ES) of *N. brasiliensis*, whereas the IgG2a antibody response against ES was negligible. On the other hand, antibody response to antigens in an extract of homogenized adult worm (AW) was observed mainly in IgG2a, with little response in IgE or IgG1. Immunohistochemical studies showed that IgE- and IgG1-binding antigens were localized almost exclusively in the subventral glands, a secretory apparatus in *N. brasiliensis*, while IgG2a-binding antigens were found mainly in the nematode wall along the body cavity. Immunoblot analysis revealed that the major IgE- and IgG1-binding molecules in ES were identical. On the other hand, some, but not all, of the major IgG2a-binding molecules in AW were different from the IgE/IgG1-binding molecules in ES. The findings suggest that the IgE/IgG1 and IgG2a antibody responses in *N. brasiliensis*-infected rats are induced by different groups of nematode antigens. Thus, it is presumed that the production of each class of antibody might be dependent, at least in part, on the nature of the antigen or antigen-linked molecules.

INTRODUCTION

It is well known that helminth infections induce remarkable specific and non-specific IgE antibody responses.[1] Recent studies have shown that, in mice, the helminth infection-induced IgE response is mediated by interleukin-4 (IL-4), and is almost completely suppressed by the injection of anti-IL-4 monoclonal antibody (mAb).[2-5] Further, analysis of CD4+ T-cell subsets in these mice showed that the helper T-cell population was skewed to Th2 cells, which produce lymphokines such as IL-4, IL-5 and IL-10.[6-8] On the other hand, the response of Th1 cells, which produce interferon-γ (IFN-γ), was down-regulated in these mice.[6-8] However, it remains to be clarified whether or not some unique molecules in the parasite constituents preferentially induce the biased Th2 response that leads to the production of IL-4 and the intense IgE antibody response.

It has previously been reported, in a study using passive cutaneous anaphylaxis (PCA) and ELISA, that the excretory/secretory product (ES) of the nematode *Nippostrongylus brasiliensis* was potently allergenic, while an antigen preparation

obtained from homogenized adult worms (AW) was less allergenic.[9] On the other hand, IgG response showed different results: the anti-AW IgG titre was much higher than the anti-ES IgG titre.[9] Thus, it was presumed that the major antigens for IgE and IgG antibody responses in *N. brasiliensis*-infected rats might be different. In this study, the target antigens were examined for IgE, IgG1 and IgG2a antibodies produced in *N. brasiliensis*-infected rats.

MATERIALS AND METHODS

Animals and sera
Specific pathogen-free, male Sprague Dawley (SD) rats were used throughout this study. Rats were subcutaneously injected with 2500 infective-stage larvae of *N. brasiliensis*, as described previously.[9] Animals were bled from the tail vein, and the sera obtained were stored at −80° until use.

Antigens
ES and AW were prepared as described previously.[9] In brief, for the preparation of ES, adult worms of *N. brasiliensis* were incubated in phosphate-buffered saline (PBS) (10,000 worms/15 ml) with 100 U/ml penicillin and 100 μg/ml of streptomycin, at 37° for 24 hr. The culture supernatant was collected, centrifuged at 11,000 *g* for 30 min and stored at −80° until use. AW was prepared by homogenizing adult worms with PBS, followed by

centrifugation at 11,000 *g* for 30 min. The supernatant was stored at −80° until use.

ELISA for antigen-specific IgE, IgG1 and IgG2a

Optimal concentrations of antigens (ES and AW) and secondary mAb were determined by testing several dilutions of each reagent. The optimal conditions employed were as follows: flat-bottomed microtitre plates (Nunc-Immunoplate; Nunc, Roskilde, Denmark) were coated overnight at 4° with 10 μg/ml of ES or AW (100 μl/well) diluted in 0·1 M carbonate buffer (pH 9·5). Washing between incubations was carried out three times, at 5-min intervals, with 0·05% Tween 20-PBS. After blocking for 1 hr with a blocking agent containing bovine milk proteins (Block Ace; Dainihon Pharm. Co., Osaka, Japan), serially diluted test sera (beginning with 1:10 dilution) were incubated for 1 hr at room temperature. After being washed, each well was applied with biotin-conjugated mouse mAb against rat IgE (MARE-1), IgG1 (MARG1-2) or IgG2a (MARG2a-8) (all diluted to 1:200; Zymed Lab. Inc., San Francisco, CA) and incubated for 1 hr at room temperature. The plates were washed, each well was applied with horseradish peroxidase-labelled streptavidin (Amersham International, Amersham, U.K.) (diluted to 1:1000), and incubation was carried out for 30 min at room temperature. After the wells were washed, a mixture of 0·04% *o*-phenylenediamine and 0·003% H₂O₂ in phosphate–citrate buffer (pH 5·0) was added to each well, and the reaction was allowed to proceed for 30 min. The reaction was stopped by adding 20 μl of 6 N sulphuric acid. The absorbance at 492 nm was measured with a microplate reader (Tosoh, Tokyo, Japan). To determine the antibody titre, the lowest dilution of test serum which gave an OD value more than twice that of normal rat serum was employed as the end-point.

Immunohistochemistry for IgE, IgG1 and IgG2a binding sites in N. brasiliensis

Adult worms were collected from the small intestine of rats, thoroughly washed in saline, and fixed with PLP fixative[10] for 4 hr at 4°. Worms were then successively placed in PBS containing 10, 15 and 20% sucrose for 4 hr, at each concentration. After freezing, cryostat sections were cut at 10 μm, and these were placed on a glass slide coated with 10% poly-L-lysin. Immunostaining of the sections was carried out with the same reagents employed for ELISA. The sections were treated with 3% H₂O₂ for 20 min at room temperature, and incubated with 25% normal rabbit serum for 1 hr at room temperature. They were then incubated with *N. brasiliensis*-infected rat serum or with uninfected normal rat serum (diluted to 1:1 for IgE, and 1:400 for IgG1 and IgG2a) at 4° overnight. After being washed, the sections were incubated with biotin-conjugated mouse mAb against rat IgE, IgG1 or IgG2a (diluted 1:100, respectively) for 1 hr at room temperature. The sections were then washed and incubated with horseradish peroxidase-labelled streptavidin (diluted 1:100) for 1 hr at room temperature. After further washing of the sections, the DAB reaction was carried out.

SDS–PAGE and immunoblotting

The ES or AW preparation (1·2 mg/ml, final concentration) was added to a solution containing 2% SDS, 10% glycerol, and 60 mM Tris buffer (pH 6·8) without reducing agents, and heated for 3 min in boiling water. SDS–PAGE and electrical transfer to a nitrocellulose membrane were carried out with a 12% gel, as described previously.[9] Immunostaining of the membrane was carried out with the same reagents employed for ELISA. After being blocked with Block Ace, the membrane was incubated at 4° overnight with *N. brasiliensis*-infected rat serum (diluted 1:1); it was then washed and incubated with biotin-conjugated mAb against rat IgE, IgG1 or IgG2a (diluted to 1:200) for 2 hr at room temperature. After being washed, the membrane was incubated with horseradish peroxidase-labelled streptavidin (1:200) for 1 hr at room temperature. The membrane was washed and the DAB reaction was carried out.

RESULTS

IgE, IgG1 and IgG2a antibody titres in rats 4 weeks after infection with *N. brasiliensis* were examined using two kinds of antigen preparations, ES and AW. As shown in Table 1, the titres of each class of antibodies against ES and AW were markedly different: the anti-ES IgE titre was 52 times higher than the anti-AW IgE titre, while the anti-ES IgG2a titre was virtually negligible compared to the significantly high anti-AW IgG2a titre. The anti-ES IgG1 titre was higher than the anti-AW IgG1 titre, although the difference was not statistically significant. The results suggest that the major target antigens for the IgE/IgG1 and IgG2a antibody responses may be different.

Next, the immunohistochemical localization of the target antigens in tissues of *N. brasiliensis* was examined. As shown in Fig. 1, IgE and IgG1 antibodies from *N. brasiliensis*-infected rats were bound almost exclusively to the subventral gland. On the other hand, IgG2a antibody binding was found mainly in the wall along the body cavity. IgG2a staining was also found in the subventral glands and gonadal cells, but it was distinctly weaker than the binding to the body wall.

ES and AW were analysed further using immunoblotting. For this purpose, SDS–PAGE was carried out only under non-reducing conditions, since it has been shown that the antigenicity of ES and AW was markedly decreased in the presence of a reducing agent.[9] As shown in Fig. 2, IgE antibody bound to multiple ES components between 24,000 and 200,000 MW. The binding pattern of IgG1 antibody was similar to that of IgE. Although IgG2a antibody bound to some ES components, the binding was weak. On the other hand, IgG2a antibody showed more distinct binding to the components in AW, especially those

Table 1. Titres of antigen-specific IgE, IgG1 and IgG2a antibodies in rats 4 weeks after infection with *N. brasiliensis*

	Antigen		Anti-ES/ anti-AW ratio
	ES	AW	
IgE	1040 ± 592	20 ± 8*	52·0
IgG1	300 ± 143	43 ± 17	7·0
IgG2a	< 20	330 ± 141*	< 0·06

Data shown are geometric means ± SEM of the end-point titres of four rats infected with *N. brasiliensis*.

* Significantly different from the corresponding value for anti-ES antibodies (*P* < 0·05 Mann–Whitney *U*-test, two-tailed).
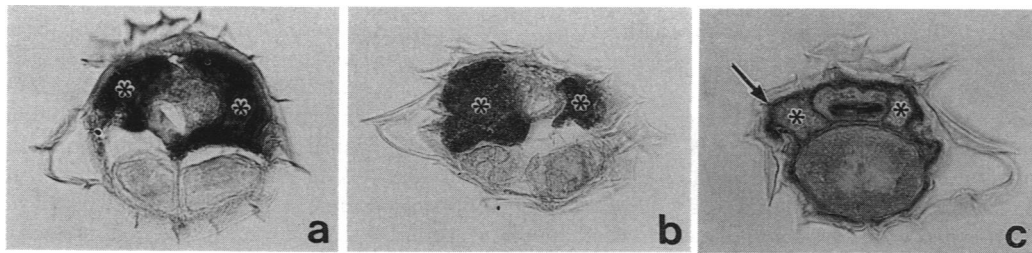
Figure 1. Immunohistochemical demonstrations of IgE (a), IgG1 (b) and IgG2a (c) antibody-binding sites in *N. brasiliensis*. Cryostat sections of adult worms were incubated with the pooled sera of five rats obtained 4 weeks after infection with *N. brasiliensis*, and the binding sites of antibodies were demonstrated with biotin-conjugated mouse mAb against rat IgE, IgG1 or IgG2a, and with horseradish peroxidase-conjugated streptavidin. Normal serum did not show any binding to the section of *N. brasiliensis*. Note that both IgE and IgG1 antibodies are almost exclusively localized in the subventral gland (*) of *N. brasiliensis*, while IgG2a antibody is found predominantly in the wall along the body cavity (arrow), and its binding to the subventral gland (*) is weak.
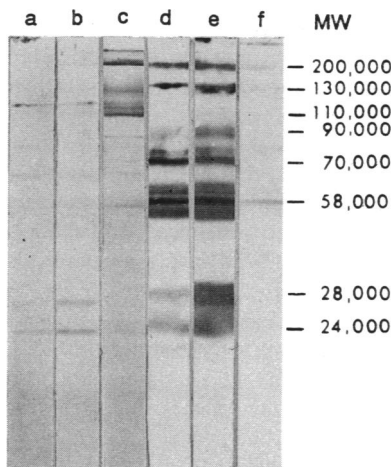

Figure 2. Immunoblot analysis of IgE, IgG1 and IgG2a binding in ES and AW. The pooled sera of five rats obtained 4 weeks after infection with *N. brasiliensis* was applied to a nitrocellulose membrane. The membrane was treated with mouse mAb against rat IgE, IgG1, or IgG2a, and then with horseradish peroxidase-conjugated streptavidin. (a) IgE binding to AW; (b) IgG1 binding to AW; (c) IgG2a binding to AW; (d) IgE binding to ES; (e) IgG1 binding to ES; (f) IgG2a binding to ES. Incubations with normal rat sera did not show any band (not shown). MW, estimated with reference to standard molecular markers (Amersham), are indicated on the right.

of more than 110,000 MW. In the IgG2a-bound molecules, at least some (e.g. 110,000 MW molecule) were not found in the IgE/IgG1-bound molecules in ES. The binding of IgE and IgG1 antibody to AW was weak.

DISCUSSION

In this study, antibody responses to infection with *N. brasiliensis* were examined using two types of antigen preparations, ES and AW. The results of the present ELISA studies showed that the titre of anti-ES IgE in *N. brasiliensis*-infected rats was significantly higher than that of anti-AW IgE, while the titre of anti-AW IgG2a was significantly higher than that of anti-ES IgG2a. IgG1 antibody responses to ES and AW were similar to IgE antibody responses. These findings suggest that IgE/IgG1 and

IgG2a antibodies are different in terms of their target antigens. The pH of the antigen coating may markedly influence the amount of antigens that bind to an ELISA plate. In our observation, the OD values of ES- and AW-specific IgE, IgG1 and IgG2a were not significantly different at the coating buffer pH of 9·5 or 7·4, but the values significantly decreased at pH 5·0 (M. Yamada & N. Arizono, unpublished data). The antigen coating at pH 9·5 in the present study seems to be adequate since the anti-ES IgE/IgG1 and anti-AW IgG2a gave high ELISA titres. It has previously been reported that the IgE ELISA was completely inhibited by preincubation of serum with homologous antigen, but not with an unrelated antigen, BSA.[9] Thus, this ELISA method is specific for the antigens and does not seem to be biased towards some limited components of antigens.

Immunohistochemical examinations showed that IgE and IgG1 binding was localized exclusively in the subventral glands of *N. brasiliensis*, which serve as a secretory apparatus in this organism.[11] This result agrees with the finding that ES, obtained from the culture supernatant of *N. brasiliensis*, included the major target antigens for IgE and IgG1. On the other hand, IgG2a antibody binding was most intense in the wall along the body cavity, although weak binding was also found in the subventral glands, as well as in gonadal cells. Thus, the results indicate that IgE and IgG1 antibody responses are induced by antigens produced in and secreted from the subventral gland of *N. brasiliensis*, but that IgG2a antibody response is induced by antigens present predominantly in the body wall.

Immunoblot analysis showed that IgE antibody bound to multiple ES components of more than 24,000 MW, as reported previously.[9] The binding pattern of IgG1 to ES was similar to that of IgE, indicating that the target antigens of IgE and IgG1 are identical. On the other hand, IgG2a antibody bound much more intensely to AW than to ES. IgG2a binding to AW was limited to antigens of relatively higher molecular weight, compared to the IgE/IgG1-binding antigens in ES. Further, at least some of the IgG2a-binding antigens were different in MW from the IgE/IgG1-binding antigens in ES.

This differing pattern of IgE/IgG1 and IgG2a antibody responses in *N. brasiliensis*-infected rats is comparable to the reciprocal regulation mechanism of IgE/IgG1 and IgG2a antibody production by IL-4 and IFN-γ, whereby IgE and IgG1 production by lipopolysaccharide (LPS)-stimulated murine B cells or by normal human B cells is enhanced by IL-4 and suppressed by IFN-γ, while IgG2a production is enhanced by

IFN-γ and suppressed by IL-4.[12-16] Further, in *N. brasiliensis*-infected mice, IgE antibody response has been reported to be suppressed by treatment with anti-IL-4 mAb.[2,3] These findings raise the possibility that the differential induction of IgE/IgG1 and IgG2a antibodies in *N. brasiliensis*-infected rats may be regulated by these cytokines.

Soloway *et al.*[17] reported that variants of synthetic peptides differed greatly in their capacity to induce hypersensitivity in mice, and that this was associated with the production of IL-4. Furthermore, HayGlass *et al.*[18-19] reported that pretreatment of mice with glutaraldehyde-polymerized ovalbumin (OVA) result in the inhibition of unmodified OVA-specific IgE response and a marked increase in anti-OVA IgG2a; the changes in IgE response induced by these modified proteins were abolished by anti-IFN-γ treatment. These findings indicate that the production of IgE and IgG2a might be dependent on the chemical and/ or physical nature of antigen, and this is probably mediated by IL-4 and IFN-γ. It has been shown that suppressor T cells also regulate the IgE antibody response.[20,21] Diaz-Sanchez and Kemeny[22,23] reported that ricin, a toxic lectin obtained from castor beans, induced selective inactivation of IgE-specific T-suppressor cells, and induced a remarkable and sustained IgE response in rats. CD8+ T cells have been shown to produce large amounts of IFN-γ.[24] Thus, if some worm antigens, or molecules physically linked to the antigens, have such effects, cytokine production could be affected.

On the other hand, it has been reported that the route of immunization also affects the induction of Th1 and Th2 cell responses; the incidence of IFN-γ-producing Th1-type cells was greater in the spleen of mice given sheep red blood cells by the systemic route, while oral immunization resulted in predominantly IL-5-producing Th2-type cells in the Peyer's patches.[25] In *N. brasiliensis*-infected rats and mice, percutaneously-infected larvae first migrate into the lung, then quickly enter the small intestine by the third to fourth day of infection.[1] Therefore, although the worm antigens are thought to be absorbed primarily through the intestinal mucosa, the possibility cannot be ruled out that particular antigen(s) are absorbed at some places along the migration route.

The differential induction of IgE/IgG1 and IgG2a antibodies by different groups of antigens in *N. brasiliensis*-infected rats may provide a model which will be useful in the analysis of the dependency of antibody response on antigen nature.

ACKNOWLEDGMENTS

REFERENCES

1. JARRET E.E.E. & MILLER H.R.P. (1982) Production and activities of IgE in helminth infection. *Prog. Allergy*, **31**, 178.
2. FINKELMAN F.D., KATONA I.M., URBAN J.F., JR, SNAPPER C.M., OHARA J. & PAUL W.E. (1986) Suppression of *in vivo* polyclonal IgE responses by monoclonal antibody to the lymphokine B-cell stimulatory factor 1. *Proc. natl. Acad. Sci. U.S.A.* **83**, 9675.
3. FINKELMAN F.D., KATONA I.M., URBAN J.F., JR, HOLMES J., OHARA J., TUNG A.S., SAMPLE J.G. & PAUL W.E. (1988) IL-4 is required to generate and sustain *in vivo* IgE responses. *J. Immunol.* **141**, 2335.
4. SHER A., COFFMAN R.L., HIENY S. & CHEEVER A.W. (1990) Ablation of eosinophil and IgE responses with anti-IL-5 or anti-IL-4 antibodies fails to affect immunity against *Schistosoma mansoni* in the mouse. *J. Immunol.* **145**, 3911.
5. URBAN J.F., JR, KATONA I.M., PAUL W.E. & FINKELMAN F.D. (1991) Interleukin 4 is important in protective immunity to a gastrointestinal nematode infection in mice. *Proc. natl. Acad. Sci. U.S.A.* **88**, 5513.
6. STREET N.E., SCHUMACHER J.H., FONG A.T., BASS H., FIORENTINO D.F., LEVERAH J.A. & MOSMANN T.R. (1990) Heterogeneity of mouse helper T cells. Evidence from bulk cultures and limiting dilution cloning for precursors of Th1 and Th2 cells. *J. Immunol.* **144**, 1629.
7. POND L., WASSOM D.L. & HAYES C.E. (1989) Evidence for differential induction of helper T cell subsets during *Trichinella spiralis* infection. *J. Immunol.* **143**, 4232.
8. PEARCE E.J., CASPAR P., GRZYCH J-M., LEWIS F.A. & SHER A. (1991) Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, *Schistosoma mansoni*. *J. exp. Med.* **173**, 159.
9. YAMADA M., NAKAZAWA M., MATSUMOTO Y. & ARIZONO N. (1991) IgE antibody production in rats against multiple components of excretory–secretory products of the nematode *Nippostrongylus brasiliensis*. *Immunology*, **72**, 104.
10. MCLEAN I.W. & NAKANE P.K. (1974) Periodate–lysine–paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* **22**, 1077.
11. LEE D.L. (1970) The fine structure of the excretory system in adult *Nippostrongylus brasiliensis* (Nematoda) and a suggested function for the excretory glands. *Tissue Cell.* **2**, 225.
12. COFFMAN R.L., OHARA J., BOND M.W., CARTY J., ZLOTNIK A. & PAUL W.E. (1986) B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J. Immunol.* **136**, 4538.
13. SNAPPER C.M., PESCHEL C. & PAUL W.E. (1988) IFN-γ stimulates IgG2a secretion by murine B cells stimulated with bacterial lipopolysaccharide. *J. Immunol.* **140**, 2121.
14. SNAPPER C.M., PECANHA L.M., LEVINE A.D. & MOND J.J. (1991) IgE class switching is critically dependent upon the nature of the B cell activator, in addition to the presence of IL-4. *J. Immunol.* **147**, 1163.
15. PÈNE J., ROUSSET F., BRIÈRE F., CHRÉTIEN I., BONNEFOY J.-Y., SPITZ H. *et al.* (1988) IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferon γ and α and prostaglandin E_2. *Proc. natl. Acad. Sci. U.S.A.* **85**, 6880.
16. PÈNE J., ROUSSET F., BRIÈRE F., CHRÉTIEN I., PALIARD X., BANCHEREAU J., SPITS H. & DE VRIES J.E. (1988) IgE production by normal human B cells induced by alloreactive T cell clones is mediated by IL-4 and suppressed by IFN-γ. *J. Immunol.* **141**, 1218.
17. SOLOWAY P., FISH S., PASSMORE H., GEFTER M., COFFEE R. & MANSER T. (1991) Regulation of the immune response to peptide antigens: differential induction of immediate-type hypersensitivity and T cell proliferation due to changes in either peptide structure or major histocompatibility complex haplotype. *J. exp. Med.* **174**, 847.
18. HAYGLASS K.T., GIENI R.S. & STEFURA W.P. (1991) Long-lived reciprocal regulation of antigen-specific IgE and IgG2a responses in mice treated with glutaraldehyde-polymerized ovalbumin. *Immunology*, **73**, 407.
19. HAYGLASS K.T. & STEFURA B. (1991) Anti-interferon γ treatment blocks the ability of glutaraldehyde-polymerized allergens to inhibit specific IgE responses. *J. exp. Med.* **173**, 279.
20. TADA T., OKUMURA K. & TANIGUCHI M. (1972) Regulation of homocytotropic antibody formation in the rat. VII. Carrier functions in the anti-hapten homocytotropic antibody response. *J. Immunol.* **108**, 1535.
21. TAKATSU K. & ISHIZAKA K. (1976) Reaginic antibody formation in the mouse. VIII. Depression of the ongoing IgE antibody formation by suppressor T cells. *J. Immunol.* **117**, 1211.

22. DIAZ-SANCHEZ D. & KEMENY D.M. (1990) The sensitivity of rat CD8+ and CD4+ T cells to ricin *in vivo* and their relationship to IgE regulation. *Immunology*, **69**, 71.

23. DIAZ-SANCHEZ D. & KEMENY D.M. (1991) Generation of a long-lived IgE response in high and low responder strains of rat by co-administration of ricin and antigen. *Immunology*, **72**, 297.

24. FONG T.A. & MOSMANN T.R. (1990) Alloreactive CD8+ T cell clones secrete the Th1 pattern of cytokines. *J. Immunol.* **144**, 1744.

25. XU-AMANO J., AICHER W.K., TAGUCHI T., KIYONO H. & MCGHEE J.R. (1991) Selective induction of Th2 cells in murine Peyer's patches by oral immunization. *Int. Immunol.* **4**, 433.