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Low-level infection with the nematode *Nippostrongylus brasiliensis* induces significant and sustained specific and non-specific IgE antibody responses in rats

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SUMMARY

Specific and non-specific IgE antibody responses were studied in SD rats infected with between 5 and 2500 *Nippostrongylus brasiliensis* (NB) larvae. In rats with 2500 NB larvae, specific IgE antibody, measured by enzyme-linked immunosorbent assay (ELISA) using NB excretory/secretory substance as antigen, reached a peak at 4 weeks of infection and gradually declined. On the other hand, in rats infected with 10 or 100 NB larvae, specific IgE was induced at 4 weeks of infection and the level continued to rise until at least 8 weeks after infection. The level at 8 weeks was significantly higher in rats infected with 10 or 100 larvae than in rats infected with 2500 larvae. The results indicate that the low-level infection induced a much more sustained specific IgE response than that induced after heavy infection. However, the level of specific IgG was correlated with the dose of infection, and reached a plateau 6 weeks after infection. Total serum IgE increased significantly even in rats infected with five larvae, a dose which did not induce detectable specific IgE. The kinetics of the production of total IgE was different in rats with light and heavy infections. In rats infected with five or 10 larvae, total IgE increased slowly and reached a plateau 4 weeks after infection. On the other hand, rats infected with more than 500 larvae showed a remarkable rise in total IgE at 2 weeks of infection; this rise gradually declined thereafter. Six weeks after infection, total IgE levels were almost the same (2–3 μg/ml) in rats infected with 10–2500 NB larvae. These results show that low-level NB infection induces a significant and sustained specific and non-specific IgE response in rats.

INTRODUCTION

It is well known that helminth infections induce remarkable specific and non-specific IgE antibody responses in humans and animals.[1] The kinetics of IgE antibody production following helminth infection has been extensively studied in rodents infected with the intestinal nematode, *Nippostrongylus brasiliensis* (NB).[2–7] Following infection with this parasite, a significant non-specific IgE response is induced, usually 2 weeks after infection. Parasite-specific IgE antibody, on the other hand, increases to a peak some 2–3 weeks later, by which time total IgE has largely declined.[5,7] These studies, however, were carried out only in rats infected with large doses of this helminth usually 2000–4000 NB. It has been shown that IgE responses are greatly affected by the immunization dose.[8–10] In general, favourable and sometimes persistent IgE responses are induced by immunization with a minute, rather than a large dose, of antigen. On the other hand, there have been few studies concerned with the effects of low-level helminth infections on IgE antibody production. In the present study, we report the IgE responses in NB-infected rats, in particular after very low-level infection with this parasite.

Abbreviation: NB, *Nippostrongylus brasiliensis*.

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MATERIALS AND METHODS

Animals and sera

Specific pathogen-free, male Sprague–Dawley rats, 7–12 weeks old, were used throughout this study. Five, 10, 100, 500, 1000 and 2500 infective-stage NB larvae were injected subcutaneously into the rats. The animals were periodically bled from the tail vein, and the serum obtained was stored at −20° until use.

ELISA assay for specific IgE, specific IgG and total IgE antibodies

Specific IgE and IgG antibodies were measured by ELISA, using adult NB excretory/secretory products as antigen, as described previously.[11] To determine total IgE antibody in rat serum, we employed an ELISA method using monoclonal anti-rat IgE mouse immunoglobulin (Zymed Lab. Inc., San Fran-

cisco, CA) and biotin-conjugated monoclonal anti-rat IgE mouse immunoglobulin (Zymed Lab. Inc.). Optimum concentrations were determined by testing several dilutions of each reagent. When monoclonal anti-rat IgE immunoglobulin or biotin-conjugated monoclonal anti-rat IgE immunoglobulin was precluded from the ELISA system, the optical density (OD) values were always less than 0·025. The procedure, carried out under optimum conditions, was as follows. The wells of a 96-well polystyrene microplate (Nunc Immunoplate, Nunc, Roskilde, Denmark) were coated with 100 μl of monoclonal anti-rat IgE (diluted to 1:100) in 0·1 M carbonate buffer (pH 9·5) overnight at 4°. Washing between incubations was carried out three times, at 5-min intervals, with 0·05% Tween 20–phosphate-buffered saline (PBS). After washing, each well was blocked for 1 hr at room temperature with a blocking reagent containing bovine milk proteins (Block Ace, Dainihon Pharm. Co., Osaka, Japan). Diluted test serum (1:10, 1:100 or 1:500) was then applied to the wells, and the plate was incubated for 2 hr at 37°. As a standard, known amounts of rat myeloma IgE (Zymed Lab. Inc.) were applied to the wells. After incubation, the plate was washed and incubated with biotin-conjugated monoclonal anti-rat IgE (diluted to 1:200) for 2 hr at 37°. The plates were then washed, and horseradish peroxidase-labelled streptavidin (Amersham, Bucks, U.K.) diluted to 1:1000 was applied to each well, following which incubation was performed for 30 min at room temperature. After washing, a mixture of 0·04% *o*-phenylenediamine and 0·003% H_2O_2 in phosphate citrate buffer (pH 5·0) was added to each well, and the plates were incubated 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). IgE antibody was detected at levels as low as 10 ng/ml. A linear relationship, up to 800 ng/ml, was observed between the OD values and the amount of myeloma IgE.

Removal of IgG antibody using protein G affinity column

A column containing 2 ml of recombinant protein G-Sepharose 4B (Zymed Lab. Inc.) was equilibrated with 0·02 M sodium phosphate buffer (pH 7·0). Two hundred microlitres of test serum was applied to the column, and the serum fraction passed through the gel was collected. For a control study, 200 μl of test serum was applied to a 2-ml column containing Sepharose CL-4B (Pharmacia LKB, Uppsala, Sweden), and the serum fraction passed through the gel was collected. The sera passed through the protein G and Sepharose CL-4B columns were adjusted to the same protein concentration, and specific IgE and IgG antibodies in these sera were assayed by ELISA.

RESULTS

Specific IgE and IgG antibody responses following NB infection

Infections with more than 10 NB induced a remarkable increase in specific IgE. In rats infected with 10 or 100 NB, titres increased at 4 weeks and continued to rise until at least 8 weeks after infection. On the other hand, the titres in rats infected with 500 or 1000 NB reached a plateau at 4 weeks of infection and did not increase further. In rats infected with 2500 NB, the level of specific IgE reached a peak at 4 weeks and decreased slightly thereafter (Fig. 1). The specific IgE level in rats infected with 2500 NB was significantly lower than that in rats infected with 10–500 NB at 6 and 8 weeks after infection ($P<0·05$, Mann–Whitney *U*-test, two-tailed).

The specific IgG level was correlated to the dose of infection; the levels were higher in heavy infections than in light infections (Fig. 1).

It is possible that excess amounts of specific IgE inhibit the binding of specific IgE to antigen and cause errors in the ELISA. Therefore, specific IgE titres were examined after the removal of IgG antibody. When IgG antibody was removed by a protein G affinity column, the OD values for specific IgG were reduced by 82–99%, and the OD values for specific IgE increased about twofold (Table 1). The rate of increase of the specific IgE titre was almost the same in rats infected with 10 and 2500 NB.

Total serum IgE levels following NB infection

Total serum IgE levels were also determined in these animals. The level at the start of infection was $0·08 \pm 0·01$ μg/ml. In rats infected with more than 500 NB, a dramatic rise in total IgE was observed 2 weeks after infection, at which time specific IgE had not yet increased (Fig. 2). The levels of total IgE in these animals declined gradually, to around 2–3 μg/ml at 6 weeks of infection. On the other hand, the increase in total IgE was slower in rats infected with 10 NB, reaching a plateau level of 2–3 μg/ml 6 weeks after infection. Total IgE also increased slowly in rats infected with five NB, a dose at which specific IgE was not detected.

DISCUSSION

Previously we reported using IgE immunoblot analysis that an excretory/secretory substance of NB contains multiple allergic components which are identical to those found in an extract from homogenized adult worms.[11] Furthermore, IgE antibody titres to the excretory/secretory substance measured by ELISA were found to be significantly higher than the titres to adult worm extract, suggesting that the former contains more allergen than the latter in the same amount of total protein. In the passive cutaneous anaphylaxis (PCA) test, similar results were obtained.[11] Therefore, our ELISA technique using an excretory/secretory substance as allergen does not seem to be biased towards some limited components of allergen.

In the present study, the threshold infection dose for the induction of parasite-specific IgE was 10 NB. The kinetics for the production of specific IgE differed according to the magnitude of infection. Low infection doses, e.g. 10 and 100 NB, induced a significant and prolonged production of specific IgE, which continued to rise until at least 8 weeks after infection. On the other hand, in rats infected with 2500 NB, specific IgE reached a peak at 4 weeks of infection, and gradually declined thereafter. It is possible that the kinetics of establishment and expulsion of NB influenced the IgE antibody response. It is well known that in rodents infected with a large number of NB, but not in animals infected with a small number of NB, rapid worm expulsion occurs between 10 and 14 days of infection.[12] However, it has also been shown that a small proportion of worms usually escape the rejection, and these worms persist for at least 2 months.[13] The size of the residual worm population has been reported to be very similar in rats with infections of between 50 and 2000 larvae.[14] In our observation, five to 77 worms were recovered 8 weeks after infection in rats infected

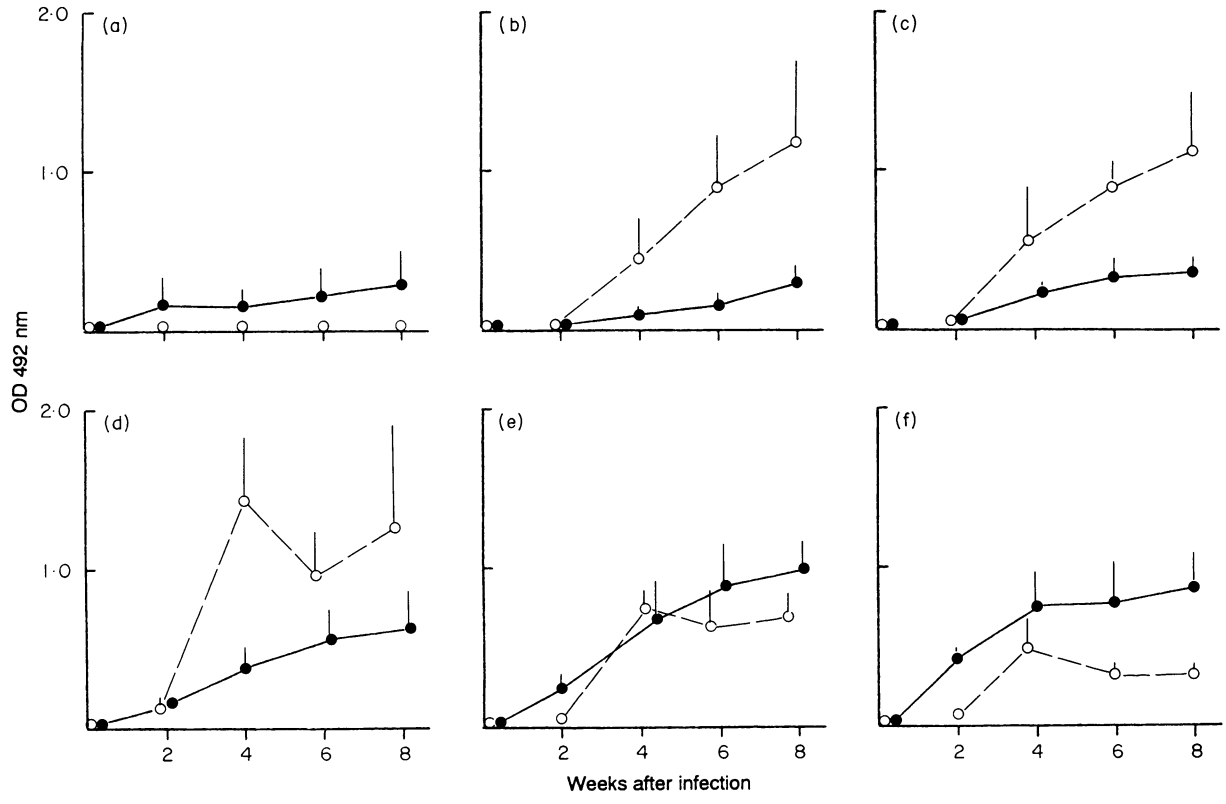

Figure 1. Parasite-specific IgE and IgG titres following infection with 5 (a), 10 (b), 100 (c), 500 (d), 1000 (e) and 2500 NB (f). The titres were determined by ELISA, using NB excretory/secretory substance as antigen. Open circles, specific IgE; closed circles, specific IgG. Data shown are the means of values from four rats. The bars at the top of the data show SD.

Table 1. Effects of IgG removal on ELISA assay of parasite-specific IgE antibody

Rat no.	No. of infective larvae	Weeks after infection	Decrease in specific IgG level after IgG removal (%)	Increase in specific IgE level after IgG removal (%)
1	2500	4	84·1	93·3
2	2500	6	82·2	110·0
3	10	4	99·0	107·1
4	10	6	99·9	88·7

Serum IgG was removed by a protein G affinity column. IgG and IgE ELISA were carried out using NB excretory/secretory substance as antigen. The percentage decrease in the OD value for specific IgG or percentage increase in the OD value for specific IgE was calculated against the values for sera passed through a Sepharose 4B column.

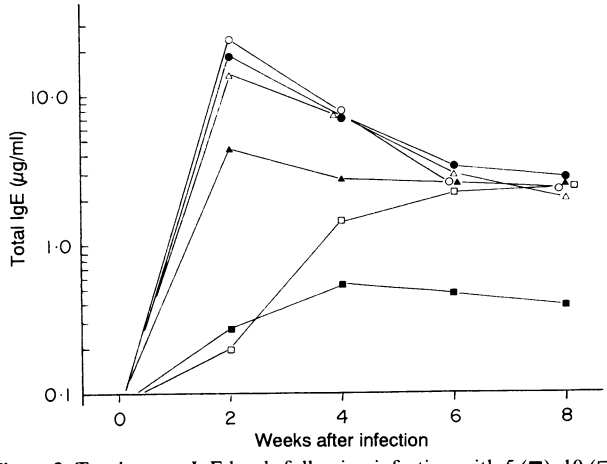

Figure 2. Total serum IgE levels following infection with 5 (■), 10 (□), 100 (▲), 500 (△), 1000 (●), and 2500 NB (○). Data shown are the means of values from four rats. The level at the start of infection, which is not shown in the figure, was 0·08 ± 0·01 μg/ml. SD which is not shown in the figure, was between 8 and 90% of the mean value.

with 1500–2500 larvae, while three to eight worms were recovered in rats infected with 10 larvae (M. Yamada, M. Nakazawa, I. Kamata & N. Arizono, unpublished data). Therefore, the present finding that high challenge doses induced a more transient and lower titre IgE response cannot be explained by the rapid expulsion of a majority of worms after a large infection. In addition, it is also possible that the circulating allergen, as well as tissue mast cells, regulates to some extent the level of specific IgE by promoting the clearance of IgE antibody. However, the titre of specific IgE started to increase at the time when most worms, except some residual worms, had been evacuated. After infection with a large number of NB, a marked increase in gut mucosal mast cells has been observed in rats.[15] However, the mast cell proliferation was observed only for a limited period, around 2 weeks of infection, and the number decreased with a half-life of 40.[16,17] In this study, mast cell number in the mucosa of the small intestine at 8 weeks of

infection was only 1·5 times higher in rats infected with 2500 larvae than in those infected with 10 larvae (data not shown). Thus, it is speculated that these factors do not markedly affect the serum level of specific IgE, at least in the later period of infection.

On the other hand, it has been reported that very high ratios of IgG to IgE cause large errors in the recovery of IgE in radioimmunoassays and ELISA of antigen-specific IgE.[18,19] Thus, it is possible that the ELISA titre of specific IgE in sera after heavy infection was greatly suppressed, since the specific IgG level was high after heavy infection. In the present study, when IgG antibody in the sera of rats infected with 2500 NB was removed by a protein G–Sepharose column, the IgE titre increased about twofold. However, specific IgE levels in sera from rats infected with 10 NB also increased about twofold. The results show that suppression of specific IgE occurred not only in sera from rats with heavy infections but also in sera from rats with light infections, in which the specific IgG level was relatively low. Therefore, the low-level specific IgE occurring in the later period of heavy infection appears to be unrelated to the degree of inhibition in ELISA.

It has been shown that immunization of rats with a low dose of ovalbumin and pertussis vaccine preferentially induces IgE antibody production, and that immunization with large doses of the antigen suppresses secondary IgE antibody responses.[8,9] Further, Vaz *et al.*[10] showed that immunization of mice with a minute dose of ovalbumin together with aluminium hydroxide gel induces persistent IgE and IgG1 antibody responses, in contrast to the higher IgG1 titre and only weak and transient IgE production induced after immunization with a large dose of the antigen. These reports coincide with results in the present study, in which we found that small infective doses induce a high and sustained IgE response, while heavy infection induces a relatively transient IgE response. It has been shown that immunization with a large dose of carrier protein together with adjuvant induces suppression of the IgE antibody response, and that this IgE suppressive effect is mediated by suppressor T cells.[20,21] Recently, Diaz-Sanchez and Kemeny[22,23] reported that the immunization of rats with an antigen and a toxic lectin, ricin, which selectively inactivates IgE-specific T-suppressor cells, induces a remarkable, sustained IgE response in both low- and high-responder rat strains. Taking these observations into account, it is not unreasonable to think that heavy NB infection also activates a suppressor mechanism.

The fact that the binding of specific IgE in ELISA was inhibited by specific IgG indicates that the antigenic molecules in excretory/secretory substances are largely identical in IgE and IgG antibodies. The parallel recognition of allergens by IgE and IgG has been reported in some human helminth infections. In filariasis and schistosomiasis, the only subclass showing consistent parallelism in antigen recognition of IgE antibodies is IgG4.[24,25] IgE- and IgG4-restricted antigen-specific responses have also been reported in atopic patients.[26] On the other hand, it has been reported that interleukin-4 (IL-4) stimulates the production of both IgE and IgG1 by murine B cells *in vitro*.[27] In several strains of mice infected with NB, the specific IgG1 serum level has been found to increase concomitantly with the rise in specific IgE.[28] However, in SJL mice, a high anti-NB titre has also been found in IgG2b.[28] In our preliminary observations in NB-infected rats, an anti-NB titre was also found in IgG2a (data not shown). Thus, it is still not clear whether IgG1 is an exclusive subclass of IgG antibodies produced in rodents after NB infection.

The IgG antibody which has the same antigenic specificity as IgE antibody has sometimes been called blocking antibody,[29] since this IgG antibody suppresses histamine release from basophils and mast cells stimulated with allergen. It has been shown that myeloma protein of the IgG4 subclass inhibits a heterologous PCA reaction in baboons that is mediated by human IgE antibodies.[30] Therefore, the production of 'blocking antibody' may have a significant effect on the allergic reaction. Our results show that heavy infection with NB induces large amounts of 'blocking antibody', while the induction of specific IgG is rather suppressed. However, the mutual roles of IgE and the blocking antibody in NB infection are not clear at present.

It is well known that a marked elevation of total serum IgE follows various kinds of helminth infections in humans and animals.[1] The level of total serum IgE has been reported to rise 2 weeks after heavy infection with NB, and to decrease gradually thereafter.[5,7] However, total IgE kinetics after light helminth infections has not yet been reported. The present study shows that infection with 10 NB induces a slow increase in total IgE; at 6 and 8 weeks, the IgE level was not significantly different from the levels in animals with higher doses of infection. These results indicate that low-level infections induce a significant and sustained increase in total IgE, although no remarkable rise occurs during the early period of infection in these animals.

The increase in total IgE after helminth infections is thought to be a polyclonal mixture to miscellaneous presensitized or unknown antigens, since the IgE titre against presensitized antigens such as ovalbumin was found to increase concomitantly with the rise in total IgE following NB infection.[4] Furthermore, absorption of serum with parasite antigens did not significantly reduce the total IgE in rats infected with NB[31] or in patients with hydatid disease, ascariasis or hookworm disease.[32,33] In the present study, total IgE was shown to increase to six times more than the control level in rats infected with only five NB, a dose at which specific IgE was not detected. Thus, our results also support the idea that the major part of increased serum IgE is not related to parasite-specific IgE.

Recently several factors which regulate IgE antibody production have been identified, including IL-4, interferon-gamma (IFN-γ) and soluble Fc RII/CD23.[34] Studies of T-cell clones established from the peripheral blood of patients with parasitic infestations have shown that these patients have significantly higher proportions of IL-4-producing, and significantly lower proportions of INF-γ-producing, $CD4^+$ T-cell clones than healthy individuals.[34] However, the parasite-derived factors which trigger and regulate specific and non-specific IgE responses have not yet been identified. These factors should be elucidated in future studies.

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