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Generation of a long-lived IgE response in high and low responder strains of rat by co-administration of ricin and antigen

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SUMMARY

Certain strains of rats infested with the nematode parasite *Nippostrongulus brasiliensis* mount vigorous, persistent immunoglobulin E (IgE) responses. In the absence of parasites, adjuvants such as *Bordatella pertussis* or $Al(OH)_3$ are needed to produce IgE responses to soluble antigens. These are short-lived, even in high IgE responder strains. In this study we have produced long-lived IgE responses in both low (Wistar) and high (Brown Norway) IgE responder strains of rats by repeated injections of ricin, a toxic lectin from castor beans, and phospholipase A_2 (PLA_2), a bee venom protein. Total IgE levels rose from 30 ± 20 ng/ml to $39{,}000 \pm 7500$ ng/ml in the Wistar rats compared with an increase from 120 ± 100 ng/ml to $47{,}000 \pm 8000$ ng/ml in the Brown Norway rats. An even greater (10^4-fold) increase was seen in PLA_2-specific IgE antibody levels. Total and PLA_2-specific IgE started to fall 6 weeks after treatment was stopped in the Wistar and after 12 weeks in the Brown Norway rats. The duration of the response was 204 and 248 days, respectively. The IgE-enhancing properties of ricin were compared in low, mid (Hooded Lister) and high IgE responder rats. Total IgE and PLA_2-specific IgE but not IgG antibody (Ab) responses were enhanced in all animals given ricin and PLA_2 but not in animals given ricin or PLA_2 alone. The increase was greater in Wistar rats (48-fold) than in Brown Norway rats (eightfold) and by Day 24 the levels of both total and PLA_2-specific IgE in the three different strains were indistinguishable. PLA_2-specific IgE antibody-secreting cells were detected in the spleen at a frequency of 1:5000. These results show: (i) that repeated immunization of rats with antigen and ricin produce a very large IgE response which was long-lived; (ii) that this response was indistinguishable in different IgE responder strains of rat; and (iii) that the IgE response declines earlier in low IgE responder strains of rats.

INTRODUCTION

Of all the immunoglobulin classes, IgE is the most tightly regulated with only a minority of individuals producing lasting IgE responses. The regulatory mechanisms that govern these responses have been studied in a number of animal models.[1–6] Of these, studies in rodents have provided the greatest insight into the regulation of the IgE isotype.[7–9] T cells have been shown to play a central role not only in the initiation of the IgE response[10,11] but also in its continued production.[12] T cells have also been shown to be potent suppressors of IgE.[13,14] T cell-derived IgE-binding factors in rats that are capable of potentiating or suppressing the nasent IgE response have been described.[15] Both factors are produced by $CD4^+CD8^-$ T cells. Other $CD4^+$ cell products have also been shown to regulate IgE, such as interleukin-4 (IL-4), which potentiates IgE in mice[16,17] and humans,[18] and interferon-gamma (IFN-γ), which antagonises this. However, other T cells may be involved as inhalation of allergen results in the generation of IgE-specific suppressor cells[19] which are $CD8^+$.[20]

In order to investigate the regulatory processes which govern IgE responses we have studied the phenomenon of castor bean allergy. Most people who are exposed to the dust of castor beans become sensitized to castor bean proteins.[21–23] These individuals produce large amounts of castor bean-specific IgE[24] and both non-atopic as well as atopic individuals become sensitized to castor bean proteins.[25] IgE responses to castor bean proteins in these individuals are long-lived even when there is no further contact with castor beans.

Injection of rats with an extract of castor bean was found to enhance the IgE response to castor bean proteins and to other antigens administered concurrently.[26] The substance in castor bean which is responsible for the enhancement of IgE is the toxic lectin, ricin. Administered together with a protein phospholipase A_2 (PLA_2) it raised both total and antigen-specific IgE levels in Hooded Lister rats.[27] Similar responses have been seen with more commonly used antigen, ovalbumin and with castor

Abbreviations: ASC, antibody-secreting cells; PBMNC, peripheral blood mononuclear cells; PLA_2, phospholipase A_2.

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bean proteins.[26] The purpose of this study was to determine whether ricin could be used to establish a long-lived IgE response and to ascertain whether this could be accomplished in low as well as high IgE responder strains of rat.

MATERIALS AND METHODS

Animals

Wistar rats (125–150 g) were purchased from Tucks (Norwich, U.K.) and Hooded Lister and Brown Norway rats (125–150 g) from Harlon-Olac Ltd (Bicester, Oxon, U.K.). No difference had previously been noted when male or female animals were used so they have not been distinguished in the text. Groups of six, age-, weight-, batch- and sex-matched, animals were used in each experiment.

Materials

PLA_2 was purified from bee venom by gel filtration in an AcA 44 column (Pharmacia LKB plc, Milton Keynes, Bucks, U.K.) and was judged to be pure by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis. Tissue culture medium RPMI-1640 was purchased from Gibco Ltd (Paisley, Renfrewshire, U.K.). Nunc maxisorb enzyme-linked immunosorbent assay (ELISA) plates were purchased from Nunc Ltd (Roskilde, Denmark). Rat serum agarose and rat IgG agarose, p-nitrophenyl phosphate, alkaline phosphatase (AP)-labelled anti-rat IgG, and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Sigma Ltd (Poole, Dorset, U.K.). Horse serum was purchased from Sera Lab (Crawley, Surrey, U.K.). Anti-rat IgG was purchased from the Binding Site (Birmingham, U.K.). Rabbit anti-rat IgE was a kind gift from the late Dr E. E. E. Jarrett (Royal Veterinary School, Glasgow, U.K.) raised against the rat IgE myeloma IR 162 and was purified by adsorption, first against rat serum agarose and then rat IgG agarose (Sigma Ltd) and finally affinity purified over rat IgE myeloma IR2 (kind gift from Professor H. Bazin, Brussels, Belgium) bound to Sepharose 4B (Pharmacia LKB plc). This anti-rat IgE (5 mg) was labelled with 5 mg of calf intestine alkaline phosphatase (Sigma) by the gluteraldehyde procedure[28] for use on the ELISA plaque assay or with Na ^{125}I (Amersham International, Amersham, Bucks, U.K.) (50/0·5 mg) by a modification of the Chloramine T method, as described previously.[29] No cross-reactivity with rat IgG, IgA or IgM was evident by gel diffusion. Purified intact ricin was a kind gift from Dr P. Thorpe (ICRF, London, U.K.). All other reagents were purchased from BHD Ltd (Dagenham, Essex, U.K.).

Immunization schedules

Schedule 1. Animals were immunized in groups of six intraperitoneally (i.p.) with 10 μg of PLA_2 in 100 μl of sterile saline. Where the protocol dictated, animals received in addition 50 ng ricin in 100 μl sterile saline i.p. at a separate site. On Day 21 booster injections of 10 μg of PLA_2 in sterile saline were given. All rats were bled from the tail under anaesthetic on Days 1, 12, 24. The serum was separated and stored at $-20°$.

Schedule 2. For the long-lived response, the dose of PLA_2 was reduced to 1 μg and the above protocol was followed up to Day 21. Thereafter, animals were injected weekly with either 5 ng ricin, 1 μg PLA_2 or 1 μg PLA_2 and 5 ng ricin and bled 3 days after each injection. Immunization was stopped after Day 63.

IgE and IgG antibodies

Specific IgE antibody to PLA_2 was measured using the radioallergosorbent test (RAST)[30] with modifications for using small volumes of serum.[31] PLA_2-coated paper discs were prepared as described previously and incubated overnight with 10 μl neat rat serum for 60 min, washed with phosphate-buffered saline (PBS) containing 0·05% Tween 20 in a washing device[32] prior to incubation for 180 min with 10 μl ^{125}I rabbit anti-rat IgE (5×10^5 c.p.m./10 μl) diluted in horse serum. The discs were washed as before and counts measured in a multichannel Gamma counter (Pharmacia LKB plc). Total serum IgE was measured as above using discs coated with anti-rat IgE and the results expressed as ng/ml by reference to the rat IgE myeloma protein IR2. The concentration of rat PLA_2-specific IgE antibodies is expressed as arbitrary units/ml by reference to a standard curve constructed using a positive serum pool. Samples which gave binding above the upper limits of the reference curve were re-assayed at 1/10 and sometimes 1/100 dilution.

IgG antibodies to PLA_2 were measured by ELISA using a modified assay for human IgG antibodies[33] that had been adapted for measuring rat IgG antibodies. Microtitre plates were coated with rabbit anti-PLA_2 in pH 9·6, 0·1 M carbonate/bicarbonate buffer overnight at 4°. All volumes were 100 μl. The plates were washed with PBS, pH 7·2 containing 0·05% Tween 20. PLA_2 was diluted to 1 μg/ml in PBS containing 0·5% Tween 20 and 0·5% horse serum (assay diluent) and incubated with the plate for 1 hr at 4°. The unbound PLA_2 was washed off as above and the serum sample, diluted 1/50 or greater, added. After 2 hr at 4° the plate was washed and AP-rabbit anti-rat IgG diluted 1/500 was added. After a further incubation of 1 hr, the plates were washed and a 1 mg/ml solution of p-nitrophenyl phosphate in pH 9·8 ethanolamine buffer was added and the absorbance read after 1·5 hr at 37° in a Titertek Multiskan plate reader (Flow Labs, Rickmansworth, Herts U.K.) at 405 nm. Results are expressed as arbitrary units/ml by reference to a standard curve constructed using a positive serum pool.

ELISA plaque assay

Spleens from treated rats were excised at Day 24 and pressed through stainless steel sieves into chilled PBS containing 0·2% bovine serum albumin (BSA), then passed through cotton wool in a 5-ml syringe barrel. The cells were then washed and 1 ml of distilled water was added, whilst whirlimixing in order to lyse the erythrocytes, and 1 ml of 1·8% NaCl was rapidly added. The cells were then washed twice in 0·2% BSA PBS and the number of viable cells determined by trypan blue exclusion. Cells were resuspended at 1×10^6 cells/ml in RPMI-1640 supplemented with 10% heat-inactivated foetal calf serum and penicillin/streptomycin. The number of IgE-secreting cells was determined using an assay adapted from that developed by Sedgwick and Holt.[34] Briefly: a microtitre plate (96-well) was coated with PLA_2 (10 μg/ml) in sodium bicarbonate buffer (pH 9·6) and incubated for 1 hr at 4°. After washing the plate with assay diluent (PBS/0·5% horse serum/0·5% Tween 20) the cells from the spleens, obtained as described above, were added (200 μl/well in RPMI/10% FCS) and incubated overnight at 37°. After washing the conjugate, AP-rabbit anti-rat IgE was added at 1/100 in assay diluent at 100 μl/well. After incubation for 3 hr at room temperature this was followed by further washing and addition of the substrate: 1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 2-amino-2-methyl-1-propanol buffer (AMP),

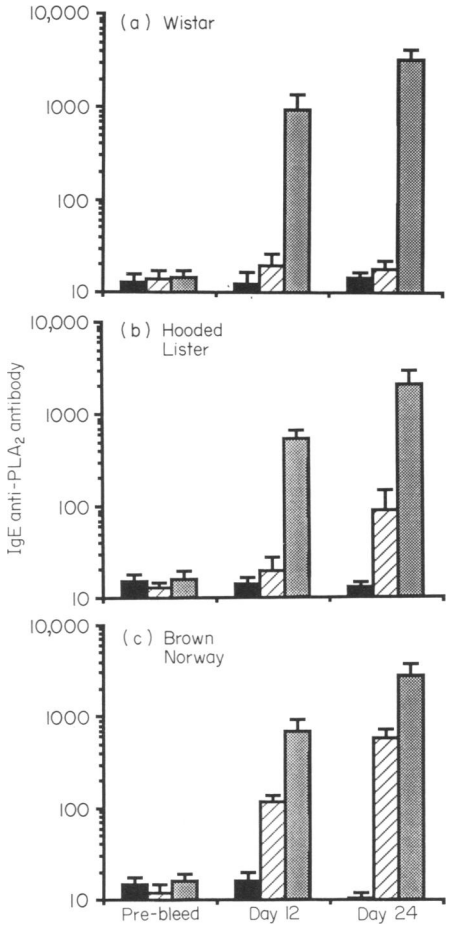

Figure 1. The IgE antibody response to PLA_2 in low (a), mid (b) and high (c) IgE responder rats immunized with saline, 10 μg PLA_2 or 10 μg PLA_2 and 50 ng ricin. The results for animals given ricin alone were the same as those given saline and so have been omitted. (■) Saline; (■) PLA_2; (▤) PLA_2 + ricin.

diluted 1 : 4 in 3% agarose, was added on a level surface at 200 μl/well. Incubation overnight at 4° allowed the spots to develop fully and the background determined by comparison with the cell-free control wells.

Statistical analysis

All analyses have been carried out on an Apple Macintosh computer using the statistical package Statview 512+. Groups were compared using the Student's *t*-test and multiple analysis of variance (MANOVA) where the data were normally distributed, and by Mann–Whitney *U*-test where they were not.

RESULTS

The effect of ricin on IgE and IgG antibody responses in different rat strains

The effect of ricin on IgE and IgG antibody (Ab) responses in different IgE responder strains of rat was compared. Animals were immunized as in schedule 1. The mean levels of IgE anti-PLA_2 antibody present in the sera of rats immunized with either saline, PLA_2 alone or ricin and PLA_2 are shown in Fig 1 for low (Fig. 1a), mid (Fig. 1b) or high (Fig. 1c) IgE responder strains.

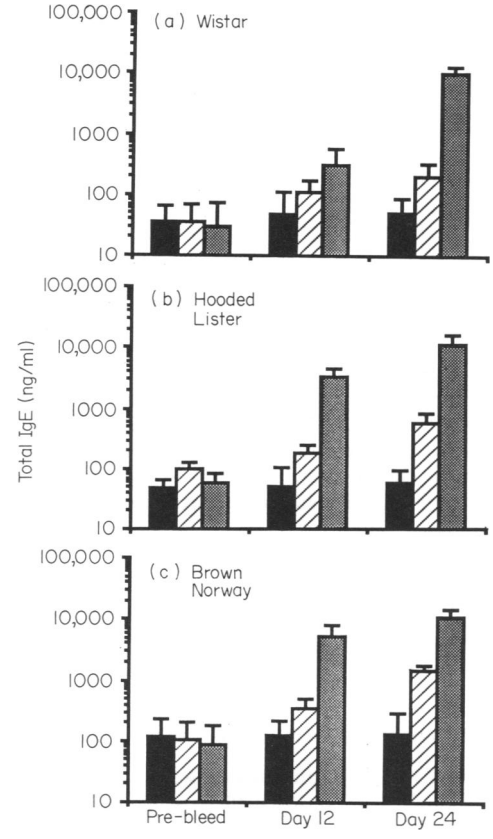

Figure 2. The levels of IgE in the sera of low (a), mid (b) and high (c) IgE responder rats immunized with saline, 10 μg PLA_2 or 10 μg PLA_2 and 50 ng ricin. The results for animals given ricin alone were the same as those given saline and so have been ommitted. (■) Saline; (■); (▤) PLA_2 + ricin.

IgE antibodies to PLA_2 were not detected in Wistar rats given PLA_2 alone, compared with a modest 10-fold rise in PLA_2-specific IgE seen in Hooded Lister rats (Fig. 1b) and a more vigorous, 100-fold rise in Brown Norway rats (Fig. 1c). These levels were significantly higher in the mid and high IgE responder strains compared to the low IgE responder strain ($P < 0{\cdot}01$ and $P < 0{\cdot}001$, respectively; paired Student's *t*-test). ByDay 24 all animals given ricin and PLA_2 showed a 1000-fold increase in the levels of IgE anti-PLA_2 antibody, which was not significantly different in any of the strains ($P > 0{\cdot}05$ Manova) either at Day 12 and at Day 24.

These sera were also assayed for their total IgE content, as shown in Fig. 2. There was a modest increase in IgE in animals given PLA_2 alone. This was seen in all three strains of rat. All animals given ricin and PLA_2 also had a greatly increased level of total serum IgE compared with control animals given saline or PLA_2 alone. Potentiation of the IgE response in animals given ricin and PLA_2 was much greater in the Wistar rats, 48-fold (Fig. 2a), than in Brown Norway rats eightfold (Fig. 2b). By Day 24 the level of total serum IgE was approximately 1000 ng/ml in all three strains of rat treated with PLA_2 and ricin. Ricin alone had no effect and the results were indistinguishable from the saline control and so have been excluded from this figure in the interests of clarity.

Although IgE antibodies were produced in all animals given PLA_2, there was no difference in PLA_2-specific or total IgG

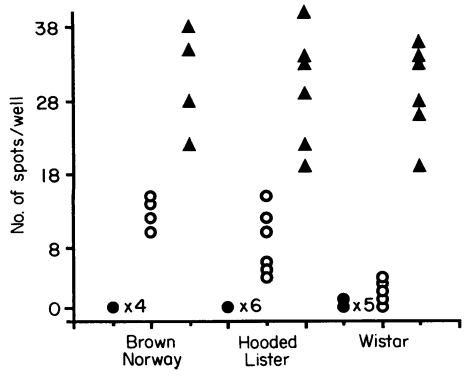

Figure 3. The number of IgE anti-PLA_2 antibody-secreting cells (ASC) detected by the ELISA plaque assay on Day 24 in the spleens of low, mid and high IgE responder rats immunized with saline, 10 μg PLA_2 or 10 μg PLA_2 and 50 ng ricin. The results for animals given ricin alone were the same as those given saline and so have been omitted. (●) Saline; (○) PLA_2; (▲) PLA_2 + ricin.

levels between those animals which were given PLA_2 and ricin and those animals given PLA_2 alone ($P > 0{\cdot}05$ Student's t-test, data not shown).

IgE-specific anti-PLA_2-secreting cells in the spleen

The animals from the groups above were killed on Day 24 and the number of IgE-secreting cells present in the spleen determined using the ELISA-plaque technique (Fig. 3). In Brown Norway rats given PLA_2 alone there were more IgE anti-PLA_2-secreting cells (70 ± 20 per 10^6 spleen cells) compared with Hooded Lister (40 ± 30 per 10^6 spleen cells) or Wistar rats (5 ± 10 per 10^6 cells). These differences were statistically significant in the case of Brown Norway or Hooded Lister versus Wistar but not Brown Norway versus Hooded Lister (MANOVA $P < 0{\cdot}01$, $P > 0{\cdot}05$). The number of IgE anti-PLA_2-secreting cells was higher in animals given PLA_2 and ricin (Brown Norway 150 ± 40; Hooded Lister 160 ± 50; Wistar 146 ± 41 per 10^6 spleen cells) and there was no statistically significant difference in the numbers found in the different strains of rat.

Duration of IgE response

The duration of the IgE response in Wistar and Brown Norway rats given immunization schedule 1 was short (Fig. 4). PLA_2-specific IgE levels had declined by Day 38 in the Wistar and by Day 52 in the Brown Norway rats (Fig. 4a). Total IgE had returned to baseline by Day 38 in the Wistar and by Day 45 in the Brown Norway rats (Fig. 4b).

Generation of a long-lived IgE response

The possibility of prolonging the IgE response by repeated immunization with ricin and PLA_2 was investigated in high and low IgE responder strains of rat. None of the animals showed any response to saline (Fig. 5) or ricin alone (not shown). Wistar rats failed to make a detectable IgE response to PLA_2 alone. In contrast, Brown Norway rats made a significant IgE antibody response but this was quickly suppressed when the animals were further immunized with PLA_2 (Fig. 4a). High and low IgE responder strains of rat receiving weekly doses of ricin and PLA_2

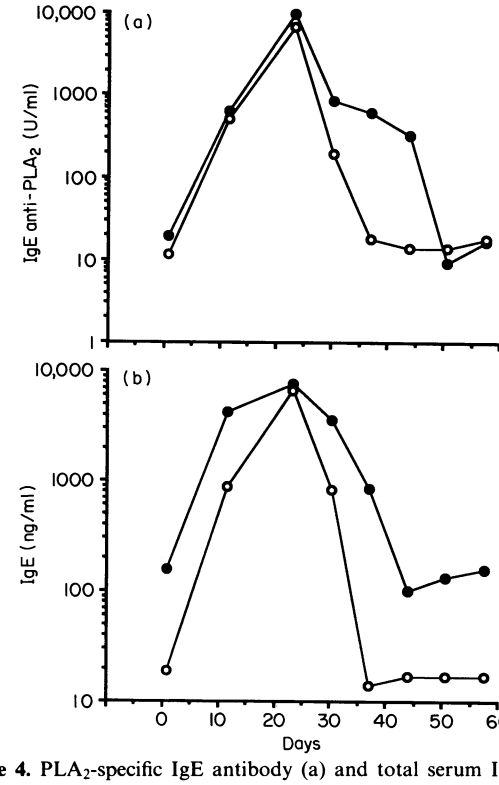

Figure 4. PLA_2-specific IgE antibody (a) and total serum IgE (b) in Wistar (○) and Brown Norway (●) rats following immunization with PLA_2 and ricin. The standard deviation has been omitted in the interests of clarity but it did not exceed 15% of the mean for total IgE or 14% of the mean for PLA_2-specific IgE.

exhibited substantial IgE responses. Total serum IgE levels rose from 30 ± 20 to $39{,}000 \pm 7500$ ng/ml in the Wistar rats compared with an increase from 120 ± 100 ng/ml to $47{,}000 \pm 8000$ ng/ml in the Brown Norway rats (Fig. 4b). An even greater (10^4-fold) increase was seen in PLA_2-specific IgE antibody levels. By day 63, when repeated exposure to ricin and PLA_2 was terminated, both strains had reached the same levels of IgE and IgE antibody, and for some time (40 days in the case of Wistar and 70 days in the Brown Norway rats) after treatment the IgE levels remained elevated. The rate of decay of the IgE response was demonstrally greater in the low IgE responder Wistar strain than in the high IgE responder Brown Norway rats, and had reached one tenth the mean plateau level in 120 days, and 160 days, respectively, and had returned to baseline in the Wistar rat by Day 204 and in the Brown Norway rats by Day 248.

DISCUSSION

Immediate-type hypersensitivity in humans is distinguished by a vigorous and persistent IgE response. Comparable long-lived IgE responses have been induced in mice[7] and in high, but not low, IgE responder strains of rat,[35] although the levels of IgE present in these animals were low. The highest levels of IgE (30–50 μg/ml) seen in rodents are produced by nematode-infested animals.[36] The levels detected in Brown Norway rats were nearly 100 times higher than those seen in Wistar rats.[32] We have previously shown that ricin, a toxin found in the beans of *Ricinus communis*, is capable of augmenting the IgE response of

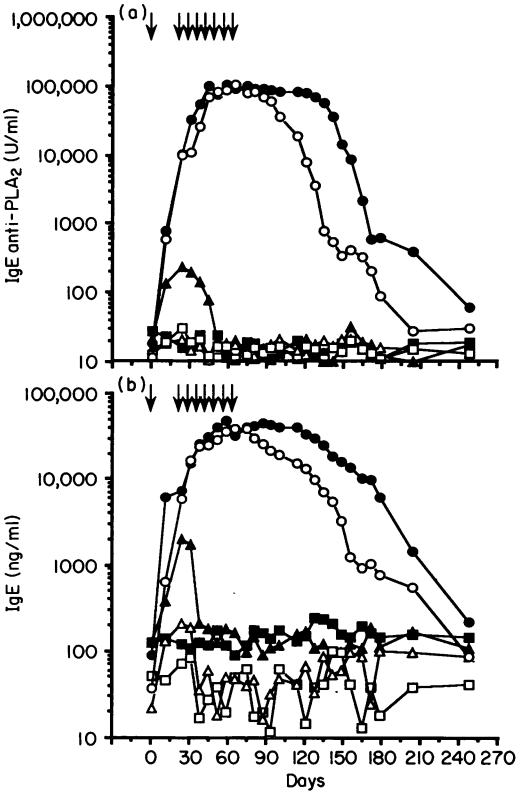

Figure 5. The levels of IgE anti-PLA_2 (a) and total serum IgE (b) in Wistar (open symbols) and Brown Norway rats (closed symbols). The primary and booster injections of PLA_2 and ricin are shown by the arrows. The standard deviation has been omitted in the interests of clarity but it did not exceed 18% of the mean for the total IgE or 17% or the mean for PLA_2-specific IgE. The results for animals given ricin alone were identical to those given saline and so are not shown. (■) Saline; (▲) PLA_2; (●) PLA_2 + ricin.

mid/high responder Hooded Lister rats to concomitantly administered antigens such as castor bean albumin, ovalbumin[26] and bee venom PLA_2.[27] In this study we investigated the effect of ricin on the IgE response of low (Wistar) and high (Brown Norway) IgE responder strains of rat. We have also studied the effect of repeated immunization with antigen and ricin on the duration of the IgE response.

Enhancement of the IgE response by co-administration of ricin and antigen was seen in all three strains of rat tested. The levels of total and antigen-specific IgE produced were identical in all three stains given ricin and antigen. In response to antigen alone, the Brown Norway rats produced a vigorous IgE response, the Hooded Lister rats a modest IgE response and the Wistar rats no IgE response. The increase in serum IgE was paralleled by an increase in the number of IgE anti-PLA_2-secreting cells in the spleen. The increase in numbers of IgE-secreting cells in ricin and antigen-treated rats implies that the adjuvant effect acts directly or indirectly on generation of IgE-secreting plasma cells. The number of these cells present in the spleen after immunization with antigen is strain dependent, but seems to be strain independent in ricin and antigen-immunized animals, further supporting the view that the ricin is over-riding the mechanism which normally regulates the generation of IgE-secreting cells in low IgE responder animals.

Repeated immunization of Wistar and Brown Norway rats with ricin and antigen produced a long-lived IgE response. Total IgE levels rose from 30 ± 20 ng/ml to $39{,}000 \pm 7500$ ng/ml in the Wistar rats compared with an increase from 120 ± 100 ng/ml to $47{,}000 \pm 8000$ ng/ml in the Brown Norway rats. The striking difference between the effect of ricin described in this paper and that of other IgE-specific enhancing agents is that the level of IgE attained by all three strains of rat is the same and is comparable with the quantity of IgE produced by parasitic infestation of high IgE responder animals. This suggests that it is a difference in the IgE suppressive mechanism which distinguishes these animals rather than a fundamental inability to make IgE. An even greater (10^4-fold) increase was seen in PLA_2-specific IgE antibody levels. Total and PLA_2-specific IgE started to fall 6 weeks after treatment was stopped in the Wistar and after 12 weeks in the Brown Norway rats. The duration of the response was 204 and 248 days, respectively.

The precise mechanism by which ricin affects IgE production is not yet clear. In contrast to more traditional IgE adjuvants, such as *B. pertussis*, and $Al(OH)_3$,[20] repeated immunization of the Wistar rat with antigen and ricin does not induce tolerance and indeed causes high IgE levels which can persist for several months without further immunization. Normally, induction of IgE tolerance by administration of soluble antigen requires doses 100–1000-fold lower in low IgE responder strains compared to high responder strains.[20] In the Wistar rat suppression of the IgE response will occur if the rat is given more than picogramme amounts of antigen, even if administered with $Al(OH)_3$.[37] With ricin, much greater amounts of antigen (1 μg of PLA_2) may be used in both high and low IgE responders to elicit an IgE response. The control group which received weekly doses of ricin alone did not show an increase of baseline IgE levels at any time, indicating that for ricin to enhance the IgE response antigen is also required, presumably to provide a stimulus for the immune system.

Other immunomodulatory drugs, such as the immunosuppressive agent cyclosporin A (CsA), can also act as IgE co-stimulators, at least in the mouse,[38] and immunosuppression by CsA has been shown to be due to specific reversible inhibition[39] through the suppression of the $CD8^+$ helper function.[40] The effects of ricin, by contrast, are lymphocytotoxic and non-reversible, possibly through the inhibition of $CD8^+$-mediated IgE isotype-specific suppressor mechanisms.[27] Both, however, seem to act preferentially on the $CD8^+$ T-cell subset at low doses. We have previously shown that $CD8^+$ T cells from untreated rats are 100 times more sensitive to the inhibitory effects of ricin than $CD4^+$ T cells.[27]

Low IgE responses in some mouse strains may be due to non-specific IgE suppression by suppressor T cells.[41,43] The same mechanism has been proposed for the rat.[31] The $w3/25^-/OX8^+$ subset is known to include T cells with apparent suppressor functions[42] and $CD8^+$ T cells generated by inhalation of ovalbumin by Brown Norway rats have been shown to mediate suppression of IgE but not IgG responses.[17] The observation that low, mid and high IgE responders reached identical levels of both total and PLA_2-specific IgE supports the view that the low IgE responder strains are normally prevented from mounting a vigorous response by a suppressive mechanism and that this is inhibited by ricin. The more rapid decline of the IgE response in the Wistar rat compared to the Brown Norway suggests a more rapid re-imposition of the suppression process in this strain.

In this study we have demonstrated that ricin, when administered to rats together with an antigen, produces an augmented IgE response. Repeated immunization with antigen and ricin results in a high level of total and antigen-specific IgE, which is sustained for 4–8 months. The same high level of IgE is attained in low, mid and high IgE responder animals treated with ricin and PLA_2. The increase in serum IgE antibody is paralleled by an increase in the number of IgE-secreting cells in the spleen to a frequently of greater than 1:10,000. The long-lived IgE response that we have established provides an ideal opportunity to study the mechanisms that regulate IgE and to investigate ways of suppressing it.

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