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Ricin enhances IgE responses by inhibiting a subpopulation of early-activated IgE regulatory CD8$^+$ T cells

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SUMMARY

Ricin, a toxic lectin from castor beans greatly enhances IgE responses to bee venom phospholipase A_2 (PLA_2) in high and low IgE responder strains of rat. The increase in IgE is accompanied by a 60% reduction in the number of CD8$^+$ but not CD4$^+$ T cells in the spleen. Optimal enhancement of IgE by ricin occurs when it is given at the same time as the antigen or 24 hr later, suggesting that it acts on cells which were activated as a consequence of immunization. Radio ligand-binding studies with ^{125}I ricin were used to compare the number of ricin binding sites on CD4$^+$ and CD8$^+$ T cells. No difference was seen in either the affinity or the number of receptors for ricin on the CD4$^+$ and CD8$^+$ T cells of unimmunized rats. In contrast, CD8$^+$ T cells taken from rats which had been immunized with 10 μg of PLA_2 24 hr earlier demonstrated considerably more ricin receptors ($3{\cdot}9 \times 10^7 \pm 2{\cdot}2 \times 10^6$ binding sites/cell) than CD4$^+$ T cells ($3{\cdot}19 \times 10^6 \pm 1{\cdot}08 \times 10^6$ binding sites/cell). However the affinity of the receptors for ricin was unchanged. Cytofluorographic analysis with fluorescein isothiocyanate (FITC)-labelled ricin confirmed these observations and indicated that increased ricin binding occurred on a subpopulation of CD8$^+$ T cells. The effect of CD8$^+$ T cells on IgE regulation was investigated by adoptive transfer. 1×10^8 highly purified (>98%) splenic CD8$^+$ T cells collected from Brown Norway rats 3 days after immunization with 10 μg of PLA_2 were adoptively transferred to naive, syngeneic recipients. The IgE antibody response to $PLA_2 + Al(OH)_3$ seen in these animals was reduced by 91%. Adoptive transfer of CD4$^+$ T cells from the same donor animals did not induce suppression and nor did adoptive transfer of CD8$^+$ T cells from animals given both ricin and PLA_2. However, when recipients of CD8$^+$ T cells taken from rats immunized with PLA_2 were immunized with a different antigen [ovalbumin (OVA)] and $Al(OH)_3$ the IgE antibody response was also suppressed, although to a lesser extent (66%). These results show that co-administration of ricin and PLA_2 depletes a subpopulation of ricin-sensitive, early activated CD8$^+$ T cells and that these CD8$^+$ T cells are potent suppressors of the primary IgE response.

INTRODUCTION

It is well established that T cells are normally required to elicit an IgE response. Neonatally thymectomized rats are unable to produce IgE unless reconstituted with thymocytes from syngeneic animals[1,2] and IgE production in parasitized mice treated with anti-CD4 antibody is suppressed.[3] T cells are also capable of suppressing primary and secondary IgE responses.[4,5] These effects are mediated, at least in part, by T-cell-derived cytokines.

Abbreviations: AP, alkaline phosphatase; Con A, concanavalin A; ELIZA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IFN-γ, interferon-γ; IL-4, interleukin-4; OVA, ovalbumin; PBS, phosphate-buffered saline; PHA, phytohaemagglutinin; PLA_2, bee venom phospholipase A_2.

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High levels of interleukin-4 (IL-4) potentiate IgE synthesis *in vitro*[6] and this can be inhibited with interferon-γ (IFN-γ) both *in vitro*[7,8] and *in vivo*.[9] IL-4 has also been shown to be required *in vivo* to maintain IgE production in both antigen and parasite-induced IgE responses.[10,11] Analysis of murine T-cell clones has shown that these cells can be subdivided according to whether they make IFN-γ and IL-2 (Th1) or IL-4, IL-5 and IL-6 (Th2)[12] although it is now clear that a number of other subpopulations of CD4$^+$ T cells with different cytokine profiles exist.[13] Little attention has been given to CD8$^+$ T cells although there is a mounting body of evidence to suggest that CD8$^+$ T cells suppress IgE. IgE-specific suppressor T cells produced by rats following inhalation of ovalbumin (OVA) were of the CD8 phenotype[14] and murine CD8$^+$ T-cell clones exhibit a similar pattern of cytokine restriction to Th1 CD4$^+$ T cells[15] although some alloreactive human CD8$^+$ T-cell clones do secrete appreciable amounts of IL-4.[16]

A role for $CD8^+$ T cells has been suggested in an animal model which we have developed in which high and long-lived IgE responses are produced following immunization of rats with antigen plus ricin.[17,18] In humans[19-24] as in rats[17,18,25] exposure to castor bean dust or ricin and antigen results in a marked potentiation of both total and specific IgE but not IgG antibody. At the height of this response the $CD4^+/CD8^+$ T-cell ratio is elevated as compared to that seen in saline-treated rats or in animals immunized with antigen alone.[18]

The potentiating effect of ricin on IgE biosynthesis has been observed with a range of antigens such as OVA, castor bean proteins[17] and bee venom phospholipase A_2 (PLA_2).[18,25] PLA_2 is used in this study as it is a well-characterized antigen. Enhanced IgE responses have been reproduced in low, moderate and high IgE responder strains of rat (Wistar, Hooded Lister and PVG, and Brown Norway) immunized with antigen and ricin.[25] Repeated boosting with ricin and antigen generates very high IgE levels (30–50 μg/ml) which persist for 9 months or more.[18]

Ricin, a potent toxin found in castor bean (*Ricinus communis*), is a 60,000 MW lectin which enters cells via the binding of one of the two galactose-binding domains on its B-chain to galactose-bearing glycoproteins on cell surfaces.[26] Additionally ricin may enter monocytes via cell surface mannose receptors.[27] Following binding the toxin is taken up by the cell and irreversibly inhibits protein synthesis by inactivating the 60S ribosomal subunit via its A-chain. It is a potent inhibitor of mitogen-induced cell proliferation *in vitro* and $CD8^+$ cells have been shown to be 100 times more sensitive to these inhibitory effects than $CD4^+$ cells.[25] What is not clear is whether the change in the ratio of $CD4^+$ to $CD8^+$ T cells *in vivo* is due to an increase in the number of $CD4^+$ T cells or to a decline in the number of $CD8^+$ T cells.[25] Also it is not clear why $CD8^+$ T cells are more sensitive to ricin than $CD4^+$ T cells. This study tested the hypothesis that ricin preferentially deletes $CD8^+$ T cells that are capable of suppressing the nascent IgE response and have investigated the cause of the greater sensitivity of $CD8^+$ T cells to ricin. Adoptive transfer has been used to provide direct evidence that the $CD8^+$ T cells are involved in the suppression of IgE. The results show that activation of $CD8^+$ T cells by antigen increases the number of ricin-binding sites present on their surface and that the inactivation of these cells leads to potentiation of the IgE response.

MATERIALS AND METHODS

Animals

Hooded Lister and specific pathogen-free (SPF) inbred Brown Norway rats (125–150 g) were bought from Harlon-Olac Ltd (Bicester, U.K.). Groups of six age, weight, batch and sex matched animals were used in each experiment.

Materials

Purified bee venom PLA_2 was a kind gift from Dr R. A. Shipolini (Imperial College, London, U.K.). Paper discs (6 mm diameter) were cut from Whatman 541 filter paper (Whatman, Maidstone, U.K.). Tissue culture medium RPMI-1640 was purchased from Gibco (Paisley, U.K.) and adjusted to 310 mOsm which is optimal for rat lymphocytes.[28] NUNC maxisorb enzyme-linked immunosorbent assay (ELISA) plates were purchased from Nunc (Roskilde, Denmark). Rat serum agarose and rat IgG agarose, *p*-nitrophenyl phosphate were purchased from Sigma (Poole, U.K.). Horse serum was purchased from Sera Lab (Crawley, U.K.). Alkaline phosphatase (AP)-labelled anti-rat IgG, IgG subclasses and IgA were purchased from The Binding Site (Birmingham, U.K.). Rabbit anti-rat IgE was a kind gift from the late Dr E. Jarrett (Glasgow, U.K.) and had been raised against the rat IgE myeloma IR162. It was adsorbed, first against rat serum agarose, then against rat IgG agarose and was finally affinity purified over purified rat IgE myeloma IR2 (kind gift from Professor H. Bazin, Brussels, Belgium) bound to Sepharose 4B (Pharmacia LKB, Milton Keynes, U.K.). There was no cross-reactivity with rat IgG, IgA or IgM as assessed by gel diffusion and less than 1% cross-reactivity in ELISA with rat immunoglobulin-coated microtitre plates. Purified ricin was a kind gift from Dr P. Thorpe (ICRF, London, U.K.) Iodobeads™ were purchased from Pierce Chemical Co. (Chester, U.K.). Monoclonal antibodies (mAb) to rat T cells were a kind gift from Drs D. Mason and N. Barclay (MRC Cellular Immunology Unit, Dunn School of Pathology, Oxford, U.K.) Fluorescein isothiocyanate (FITC)-ricin was from Sigma. All other reagents were purchased from BDH Ltd (Dagenham, U.K.).

Fractionation of lymphocyte subpopulations

Spleens from rats were excised and pressed through stainless steel sieves into chilled phosphate-buffered saline (PBS) containing 0·2% bovine serum albumin (BSA), then passed through sterile cotton wool in a 5-ml syringe barrel. The eluted cells were then washed twice with 0·2% BSA in PBS. Contaminating erythrocytes were lysed by hypotonic treatment—1 ml of distilled water was added to the cells whilst whirlmixing, immediately followed by 1 ml of 1·8% NaCl to restore osmolarity. The cells were then washed twice in 0·2% BSA in PBS and the number of viable cells determined by trypan blue exclusion.

The T-cell subpopulations were depleted of macrophages by adherence and positively selected by panning:[29] 100 × 15 mm Petri dishes (Sterilin, Hounslow, U.K.) were coated with 8 ml of PBS containing 10 μg/ml of either MRC OX8 (anti-CD8) or w3/25 (anti-CD4) antibody and left overnight at 4°. The solution was then decanted and the plate washed twice with 5 ml of PBS. Finally 5 ml of 0·2% BSA PBS was added and the Petri dishes left to stand at room temperature for 30 min. The Petri dishes were washed once with PBS and the cells added at 2×10^7 cells/dish in 4 ml 0·2% BSA PBS and incubated for 25 min at 4°, the dish was then gently swirled once and incubated for a further 20 min. Non-adherent cells were decanted and remaining adherent cells washed three times with 5 ml cold 0·2% BSA PBS. Adherent cells were collected using xylocaine which was added at 4 mg/ml, left for 15 min at room temperature and followed by 40 ml of cold 0·2% BSA PBS and forceful pipetting to dislodge the cells. The cells were washed, counted and the purity of all preparations checked by flow cytofluorographic analysis in a FACScan (Becton Dickinson, Abingdon, U.K.). Cell preparations were rejected if they were less than 98·5% pure for CD4 or CD8. The mAb used were: MRC OX8 directed against rat CD8 antigen;[30] w3/25 directed against rat CD4 antigen;[31] MRC OX21 directed against human C3b inactivator but not rat cells[32] and MRC OX1 the anti-leucocyte common antigen.[33] The purity of T cells was determined using the mAb OX19 which recognizes a pan-T marker CD5. Greater than 98% of the pure CD4 and CD8 positive cells were T cells.

Immunization schedule

Hooded Lister rats were immunized in four groups of six intraperitoneally (i.p.) with 10 μg of the antigen (PLA_2) in 100 μl of sterile saline with or without ricin. For the experiments on the time dependency of ricin on IgE antibody responses, all animals were immunized with 10 μg of PLA_2 and were also given 50 ng of ricin in 100 μl sterile saline i.p. at a separate site 24 or 72 hr before, simultaneously, or 24 or 72 hr after PLA_2. The control group received no ricin. On day 21 booster injections of 10 μg of PLA_2 in 100 μl of sterile saline were given. All rats were bled from the tail under Halothane anaesthetic on days 1, 12 and 24. The serum was separated and stored at $-20°$.

IgE antibodies

Specific IgE antibody to PLA_2 and total serum IgE were measured using the micro-radioallergosorbent (RAST) and radioimmunosorbent (PRIST) tests, respectively, as previously described.[25,34] Briefly, cyanogen bromide-activated paper discs (2 g Whatman 541) were coated with a 100 μg/ml solution of OVA or PLA_2 for the RAST or with 100 μg/ml of anti-rat IgE for the PRIST as described previously.[34] The discs were incubated with 10 μl of rat serum for 60 min, washed with PBS containing 0·05% Tween 20 and incubated 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 counted in a multichannel γ-counter (Pharmacia). Total serum IgE was expressed as ng/ml by reference to the rat IgE myeloma protein IR2. The concentration of rat PLA_2-specific IgE antibodies was expressed as arbitrary units/ml by reference to an in-house reference serum pool. Samples which gave binding above the upper limits of the reference curve were reassayed at 1/10 and sometimes 1/100 dilution until they fell within the limits of the curve.

IgG antibodies

IgG antibodies to PLA_2 were measured by ELISA using a modified assay for human IgG antibodies[35] that had been adapted for 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 10 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 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, U.K.) at 405 nm. Results are expressed as arbitrary units/ml by reference to a standard curve constructed using a positive serum pool.

Cytofluorographic analysis

1×10^6 lymphocytes were labelled for 45 min with 10 μl of the appropriate mAb conjugated to phycoerythrin (Serotec, Oxford, U.K.) or with 10 μl of a 1/20 solution of ricin conjugated to FITC (Sigma) and diluted in 0·2% BSA/PBS with 10 mM sodium azide. 10^4 cells were analysed on a FACScan.

^{125}I radiolabelling of ricin

Ricin was radiolabelled using Iodo-bead™. Ten micrograms of toxin in 100 μl of PBS (pH 7·4, 0·1 M) was added to one Iodo-bead™ and allowed to react with 1 mCi (10 μl) of Na ^{125}I (Amersham) for 5 min at room temperature. The reaction was stopped by the addition of 100 ml of 0·1 M KI. The iodinated ricin was purified by gel filtration using a 10-ml Bio-gel P-30 column (Bio-Rad, Watford, UK; exclusion limit 30,000 MW). Radiolabelled ricin was then adjusted to a specific activity of 2×10^4 c.p.m./μg by addition of unlabelled ricin. Both native and iodinated ricin inhibited mitogen-induced proliferation of splenic mononuclear cells equally indicating that the radiolabelling procedure had not altered its biological activity.

Binding of ricin to CD4$^+$ and CD8$^+$ T cells

Binding of ricin to lymphocytes was measured by incubating 5×10^6 purified CD4$^+$ or CD8$^+$ T cells from rat spleens in 5 ml Hanks' balanced salt solution (HBSS) (10 mM HEPES, 0·1% BSA, 0·2% NaN_3) with a fixed concentration of ^{125}I-radiolabelled ricin (2×10^4 c.p.m./μg, $2{\cdot}5 \times 10^6$ c.p.m./ml) for defined periods of time on a rocker (15–150 min). For the Scatchard plots, the tubes were incubated for 60 min with different concentrations of ^{125}I-radiolabelled ricin. The cells were pelleted by centrifugation at 300 *g* for 7 min at 4°. Duplicate 1 ml aliquots of supernatant were then removed and the amount of free ricin in each supernatant determined by measuring the radioactivity in a multichannel γ-counter (Pharmacia). Additionally, the pellet was resuspended in 1 ml HBSS and counted as a control. Background and non-specific binding were determined by assaying the amount of radioactive ricin bound in the presence of 100-fold excess of cold ricin of each different concentration of ^{125}I-radiolabelled ricin.

Adoptive transfer

Groups of four Brown Norway donor rats were given either: 100 μl saline, 10 μg PLA_2 or 10 μg PLA_2 and 50 ng ricin. Three days later they were killed and their spleens excised and cells prepared as follows: CD8$^+$ T cells were isolated by depletion of cells expressing CD4 (w3/25), major histocompatibility complex (MHC) class II (MRC OX6) and surface Ig (MRC OX12) by panning and any residual contamination was removed using mAb followed by sheep anti-mouse Ig-coated magnetic beads (Dynal AS, Oslo, Norway). Similarly, CD4$^+$ T cells were isolated by depletion of cells expressing CD8 (MRC OX8), MHC class II and surface Ig by panning. Additionally macrophages were removed by panning using MRC OX43. Any residual contamination was removed as above. 1×10^8 CD8$^+$ cells or CD4$^+$ T cells were introduced intravenously into each recipient. One hour later the recipient Brown Norway rats were bled, then immunized with 10 μg PLA_2 and 100 μg $Al(OH)_3$. After 21 days the rats were boosted with 10 μg of PLA_2 alone and after 24 days the rats were bled and the total and PLA_2-specific IgE levels determined.

Statistical analysis

All analyses have been carried out on an Apple Macintosh computer using the statistical package STATVIEW 512+. Groups were compared using a Mann–Whitney *U*-test, since some of the data showed a skewed distribution. Binding affinities and the number of receptors were calculated using Scatchard plots with the LIGAND computer program.[36]

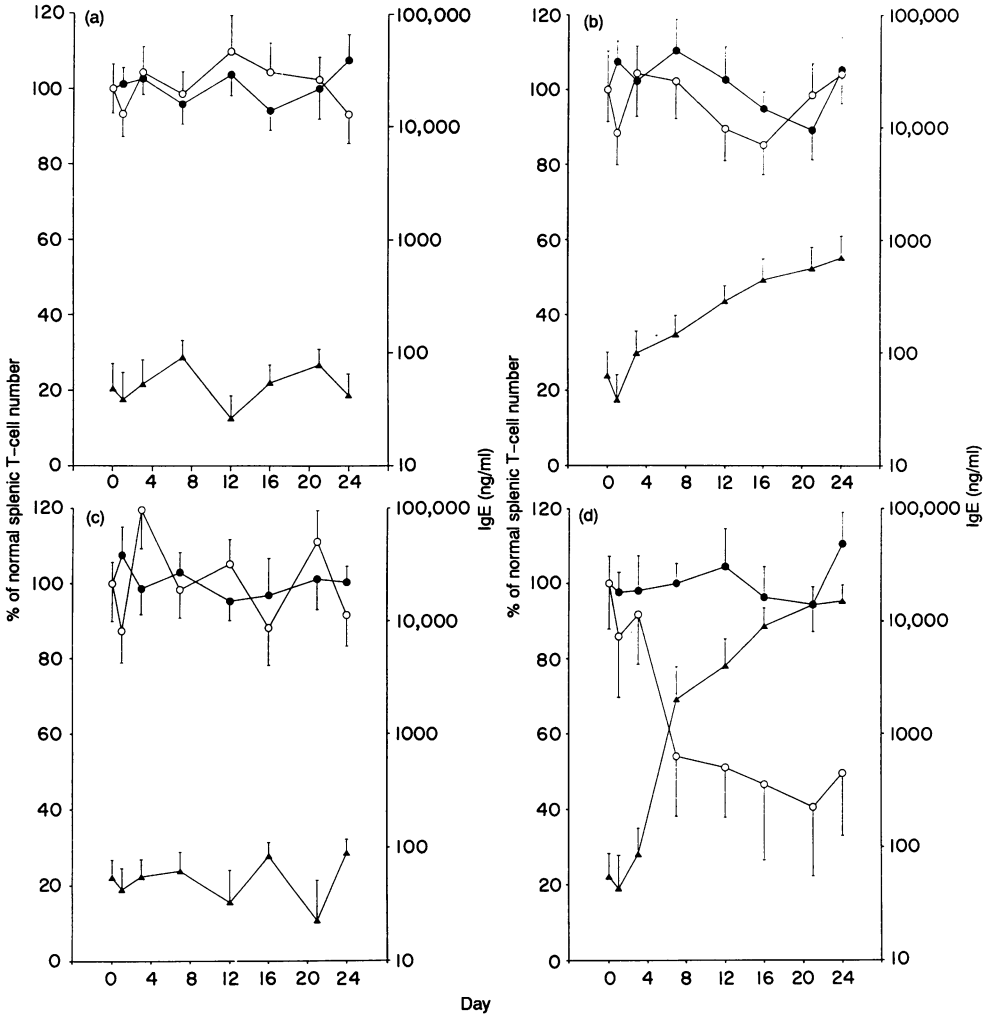

Figure 1. The mean level of IgE (▲) and the number of $CD4^+$ (●) and $CD8^+$ (○) T cells in the spleens of rats immunized with (a) saline, (b) 10 μg PLA_2, (c) 50 ng ricin and (d) 10 μg PLA_2 + 50 ng ricin expressed as per cent of initial values. Error bars indicate the standard deviation.

RESULTS

In vivo changes in splenic $CD4^+$ and $CD8^+$ T-cell numbers following immunization with ricin and antigen

The relationship between total serum IgE levels and the numbers of $CD4^+$ and $CD8^+$ T cells in the spleen was determined at various times following immunization. In rats given saline (Fig. 1a) there was no significant difference in either the level of serum IgE or the number of $CD4^+$ or $CD8^+$ cells ($P > 0.05$) in the spleen on day 24 as compared to preimmunization. Mean serum IgE levels in rats immunized with PLA_2 alone (Fig. 1b) increased from 62 ng/ml before immunization to 698 ng/ml 24 days after immunization. $CD4^+$ and $CD8^+$ T-cell numbers before and after immunization with PLA_2 were not significantly different at any time-point ($P > 0.05$). Furthermore, there was no statistically significant change in $CD4^+$ or $CD8^+$ T-cell numbers or in the level of serum IgE (mean 55 ng/ml) in rats given ricin alone (Fig. 1c).

The number of $CD8^+$ T cells in the spleens of rats immunized with both ricin and PLA_2 (Fig. 1d) declined 7 days after immunization ($P < 0.01$) and reached a low point, 41% of the starting $CD8^+$ T-cell number, 21 days after immunization. The number of $CD4^+$ cells present did not vary significantly ($P > 0.05$). The decline in $CD8^+$ T-cell numbers was paralleled by the increase in mean total serum IgE from resting levels of 56 ng/ml to 15,062 ng/ml 24 days after immunization.

Effect of timing of antigen and ricin on IgE responses

Rats were immunized with 10 μg of PLA_2. In addition, ricin was also administered 24 or 72 h prior to the PLA_2, at the same time as the PLA_2 or 24 or 72 hr later. The same pattern was observed in the primary (day 12) and secondary (day 24) responses, although the differences were more marked in the latter (Fig. 2a, b). At day 24 there was a significant IgE response to PLA_2 in all groups ($P < 0.01$) (Fig. 2a). The highest levels attained were in those rats which had been given ricin and PLA_2 at the same time (mean 2213 U/ml) or those animals to whom ricin had been given 24 hr after the PLA_2 (mean 3120 U/ml). The levels of PLA_2-specific IgE antibody in these animals were significantly greater ($P < 0.005$) than those found in rats immunized with PLA_2 alone (mean 91 U/ml). However, the IgE response in rats

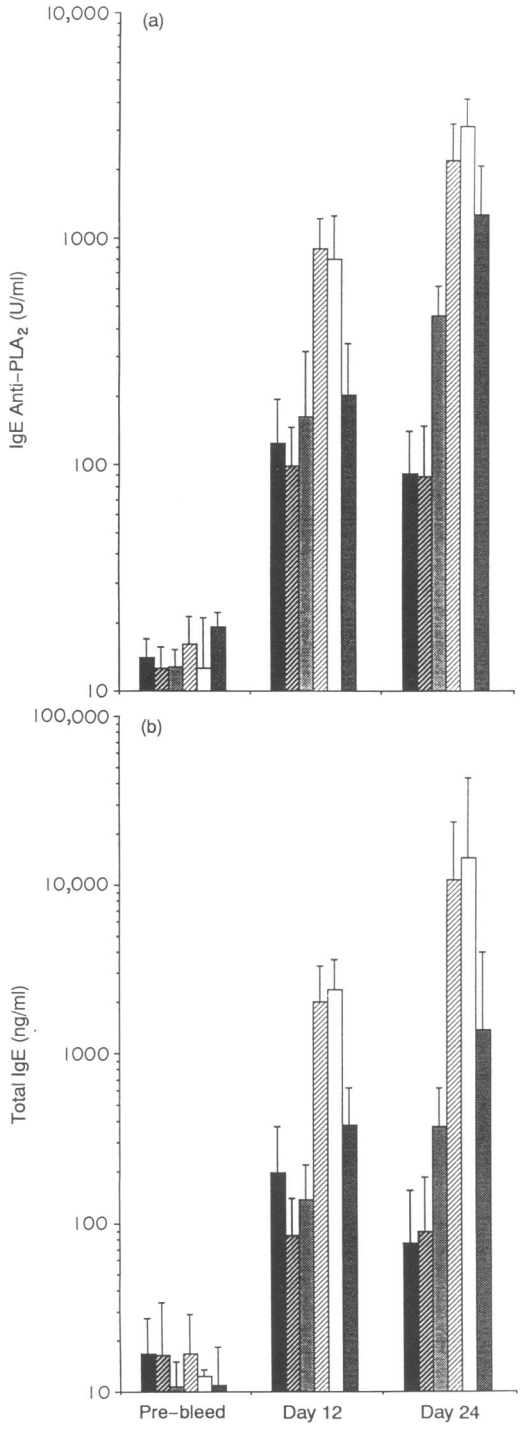

Figure 2. The mean levels of IgE antibody to PLA_2 (a) and total serum IgE (b) in the sera of rats immunized with 10 μg PLA_2 alone (■), or 10 μg PLA_2 and 50 ng ricin administered 72 (▨) or 24 hr (▩) earlier, together with the PLA_2 (▨) or 24 (□) or 72 hr (■) later. Error bars indicate the standard deviation.

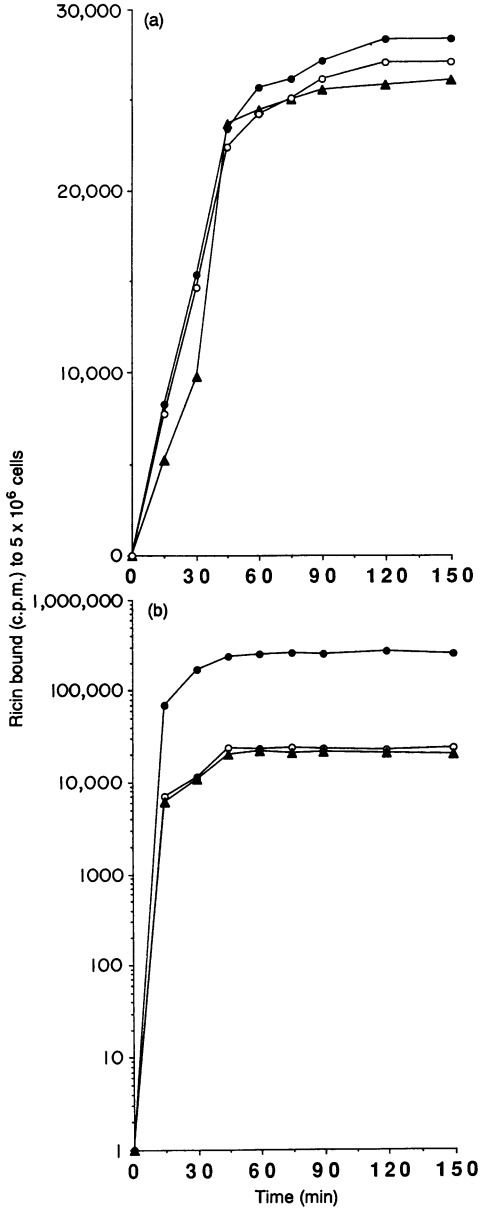

Figure 3. Time-course of [^{125}I]ricin binding to purified $CD4^+$ (▲), $CD8^+$ (●) and unfractionated (○) splenic T cells from (a) naive rats and (b) from rats immunized 24 hr earlier with 10 μg of PLA_2. Cells were cultured with a fixed concentration of radiolabelled ricin (1×10^4 c.p.m./μg, $2{\cdot}5 \times 10^6$ c.p.m./ml).

given ricin 72 hr after immunization with PLA_2 (mean 1261 U/ml) was still potentiated as compared to rats immunized with PLA_2 alone ($P < 0{\cdot}01$). In contrast, in those rats exposed to ricin 24 hr prior to immunization, the IgE response was weaker (mean 456 U/ml) and potentiation less ($P < 0{\cdot}05$). If the ricin was given 72 hr before the PLA_2 the IgE response (mean 89 U/ml) was not significantly different to that seen when rats were immunized with PLA_2 alone ($P > 0{\cdot}05$).

Total serum IgE levels closely followed antigen-specific IgE antibody titres (Fig. 2b) and rose in all animals ($P < 0{\cdot}01$). When ricin was administered 24 hr after PLA_2 a very high level of serum IgE (mean 14,599 ng/ml) was seen, comparable to that found when ricin and antigen were administered together (mean 10,780 ng/ml). Both were significantly greater ($P < 0{\cdot}001$) than the IgE levels seen in rats immunized with PLA_2 alone (mean 76 ng/ml) and, indeed, when ricin was given 72 hr after antigen, serum IgE (mean 1395 ng/ml) was also increased ($P < 0{\cdot}01$). However, when ricin was administered 72 hr before the antigen,

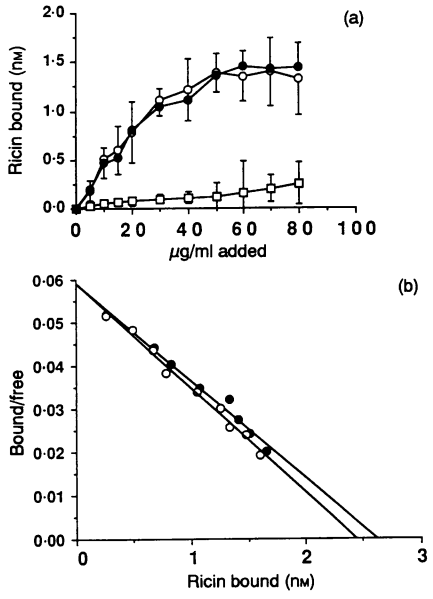

Figure 4. [^{125}I]ricin binding to 5×10^6 purified CD4$^+$ and CD8$^+$ T cells from unimmunized rats. Increasing quantities of [^{125}I]ricin were incubated with the cells for 60 min. Saturation binding (a) of [^{125}I]ricin to purified CD4$^+$ (○) and CD8$^+$ (●) splenic T cells from naive rats. The data are expressed as the mean and standard deviation. Controls for non-specific binding to purified CD4$^+$ and CD8$^+$ splenic T cells to which a 100-fold excess of cold ricin had been added. (b) Scatchard plot of binding of [^{125}I]ricin to the same cells.

no significant difference in the IgE levels (mean 90 ng/ml) was evident as compared to rats immunized in the absence of ricin ($P > 0{\cdot}05$). When ricin was given 24 hr before the antigen a modest increase in total IgE (mean 412 ng/ml) was seen ($P < 0{\cdot}05$). When ricin alone was given no increase in IgE was observed (Fig. 1c).

Binding of [^{125}I]ricin to T-lymphocyte subpopulations

In order to determine the reason for the greater sensitivity of CD8$^+$ T cells to ricin, radioligand-binding studies were performed in unimmunized and immunized rats. Binding of [^{125}I]ricin to purified CD4$^+$ and CD8$^+$ T cells reached plateau levels after 45 min at 37°. Identical results were seen with T cells from the spleens of both unimmunized rats (Fig. 3a) or rats which had been immunized with 10 μg of PLA_2 24 hr earlier (Fig. 3b).

Saturation curves (Fig. 4a) and Scatchard plots (Fig. 4b) for CD4$^+$ and CD8$^+$ T cells derived from the spleens of unimmunized rats were very similar and no differences in binding constants were detected ($P > 0{\cdot}05$). The total number of ricin-binding sites (Table 1) was essentially the same on CD4$^+$ (mean $2{\cdot}9 \times 10^6$/cell) and CD8$^+$ (mean $3{\cdot}2 \times 10^6$/cell) T cells. Furthermore, there was no difference in the affinity with which ricin bound to the different cell types. The mean K_a was $2{\cdot}4 \times 10^7$ for CD4$^+$ T cells and $2{\cdot}3 \times 10^7$ for CD8$^+$ T cells.

CD8$^+$ T cells derived from spleens of rats immunized with 10 μg of PLA_2 24 hr earlier yielded saturation (Fig. 5a) and Scatchard plots (Fig. 5b) which differed markedly from those obtained with CD4$^+$ cells (Fig. 5c, d). The number of binding sites (Table 1) on CD8$^+$ T cells was much greater (mean $3{\cdot}9 \times 10^7$) compared with CD4$^+$ T cells (mean $3{\cdot}2 \times 10^6$) although the affinity constant did not differ (mean K_a $2{\cdot}3 \times 10^7$ for CD4$^+$ T cells and $2{\cdot}4 \times 10^7$ for CD8$^+$ T cells).

Table 1. Binding of [^{125}I]ricin to T-lymphocyte subpopulations. The binding constant of ricin (K_a) and the number of ricin-binding sites/CD8$^+$ and CD4$^+$ T cells derived from the spleens of unimmunized rats and rats which had been immunized with 10 μg of PLA_2 24 hr earlier

	Binding constant K_a (M^{-1})	No. of binding sites/cell
Unimmunized		
CD8$^+$	$2{\cdot}26 \times 10^7 \pm 1{\cdot}26 \times 10^6$	$3{\cdot}16 \times 10^6 \pm 1{\cdot}76 \times 10^6$
CD4$^+$	$2{\cdot}42 \times 10^7 \pm 2{\cdot}14 \times 10^6$	$2{\cdot}94 \times 10^6 \pm 1{\cdot}43 \times 10^6$
Immunized		
CD8$^+$	$2{\cdot}42 \times 10^7 \pm 3{\cdot}2 \times 10^6$	$3{\cdot}92 \times 10^7 \pm 2{\cdot}21 \times 10^6$
CD4$^+$	$2{\cdot}34 \times 10^7 \pm 1{\cdot}01 \times 10^6$	$3{\cdot}19 \times 10^6 \pm 1{\cdot}08 \times 10^6$

Immunofluorescence staining

The binding of ricin to highly purified CD4$^+$ and CD8$^+$ T cells isolated from the spleens of naive rats and rats which had been immunized 24 hr earlier with 10 μg of antigen was studied further by cytofluorometry with FITC ricin. CD8$^+$ T cells from naive rats (Fig. 6) bound ricin with equal intensity to CD4$^+$ T cells. Following immunization with PLA_2 a shift to the right was seen in the immunofluorescence profile of the CD8$^+$ T cells indicating increased binding of ricin to the cell surface. No increased binding could be seen with the CD4$^+$ T cells. Similar results were observed when the rats were immunized with OVA; CD8$^+$ T cells showed increased binding, whilst there was no change in CD4$^+$ T cells.

Adoptive transfer of CD8$^+$ T cells

The effect of ricin-sensitive CD8$^+$ T cells on the IgE response was studied in naive, syngeneic recipients by adoptive transfer. Brown Norway rats were used as they produce appreciable and reproducible IgE responses when immunized with antigen and $Al(OH)_3$. CD8$^+$ T cells (99% CD8$^+$ and >98·5% CD5$^+$) were adoptively transferred from rats which 3 days before had been given saline or had been immunized with PLA_2 alone or with PLA_2 plus ricin, to naive syngeneic recipient rats which were then immunized with PLA_2 and $Al(OH)_3$. Those rats which received CD8$^+$ T cells from donor rats injected with saline had PLA_2-specific IgE levels (mean 1027 U/ml) (Fig. 7a) and total serum IgE levels (mean 3546 ng/ml) (Fig. 7b) which were significantly higher than was detected preimmunization (mean 15 U/ml and 159 ng/ml respectively ($P < 0{\cdot}001$ for both). By contrast, in rats which received CD8$^+$ T cells from donor animals injected with PLA_2 alone, the PLA_2-specific IgE response was reduced by 91% (mean 90 U/ml) and total serum IgE levels by 92% (mean 286 ng/ml) and were not significantly greater than preimmunization ($P > 0{\cdot}05$). Rats receiving CD8$^+$ T cells derived from donor animals immunized with both ricin

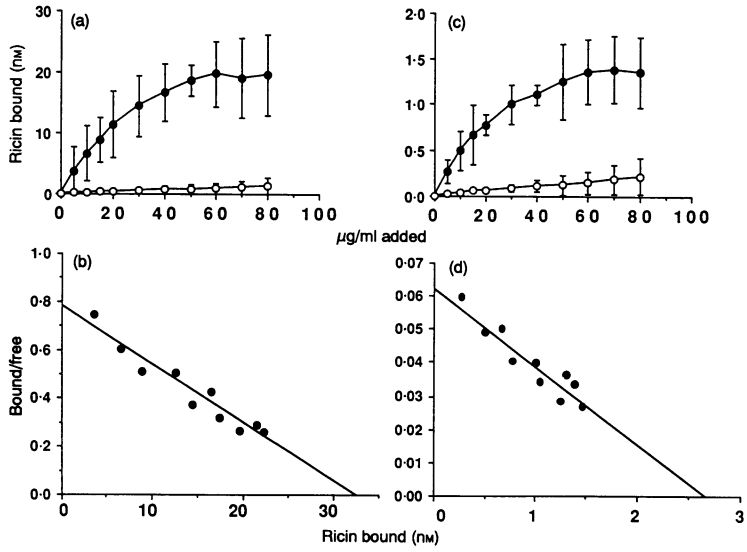

Figure 5. Ricin binding to CD4+ and CD8+ T cells from immunized rats (10 μg PLA_2). Increasing doses of [^{125}I]ricin were incubated with the cells for 60 min. Saturation binding of [^{125}I]ricin to (a) purified CD8+ and (c) CD4+ splenic T cells. The data are expressed as the mean and standard deviation. Scatchard plot (b and c) of binding of [^{125}I]ricin to purified CD8+ (b) and CD4+ (d) splenic T cells. Closed symbols are the tubes to which cells and [^{125}I]ricin had been added. Open symbols represent controls for non-specific binding to which a 1000-fold excess of cold ricin had been added.

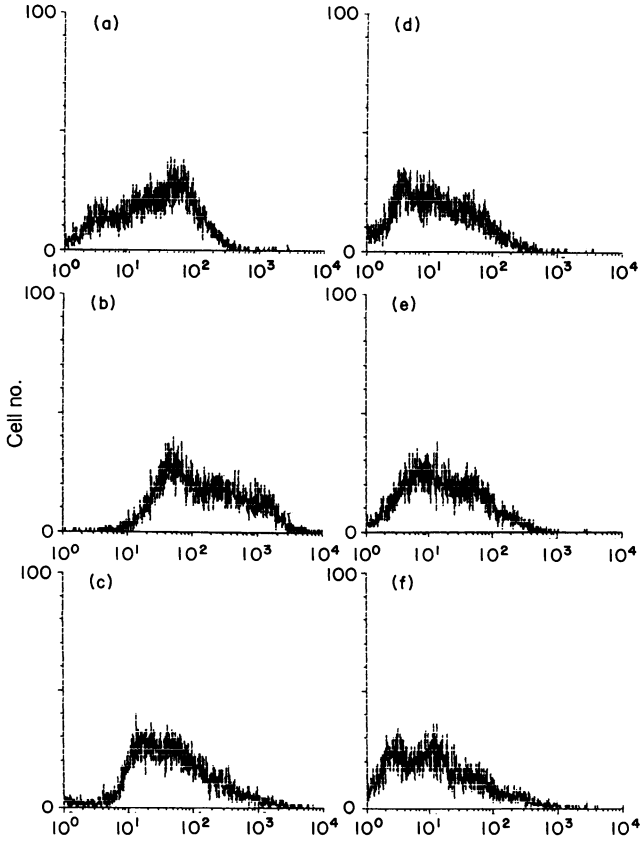

Figure 6. Cytofluorographic analysis of FITC ricin binding to purified CD8+ (a–c) and CD4+ splenic T cells (d–f) from rats which had been injected with either saline (unimmunized) or 10 μg PLA_2 or 10 μg OVA 24 hr beforehand.

and antigen had antigen-specific IgE (mean 1513 U/ml) and total IgE levels (mean 4142 ng/ml) which were similar to those seen in rats receiving cells from saline-treated donors.

PLA_2-specific IgG levels (Fig. 7c) were similar in rats which received CD8+ T cells from donor rats given saline (mean = 211 U/ml), PLA_2 alone (mean 170 U/ml) or PLA_2 plus ricin (mean 155 U/ml). These levels were significantly greater than preimmunization ($P < 0.001$ for all three groups).

Adoptive transfer of CD4+ T cells

The effect of adoptive transfer of CD4+ T cells on the IgE response was also examined. The procedure above was repeated except that 1×10^8 CD4+ T cells (>99% CD4+ and >97% CD5+) were adoptively transferred. PLA_2-specific IgE levels (mean 1316 U/ml) (Fig. 7d) and total serum IgE levels (mean 3313 ng/ml) (Fig. 7e) were significantly higher than preimmunization (mean 18 U/ml and 139 ng/ml respectively) ($P < 0.001$ for both) in rats which received CD4+ T cells from donor animals injected with saline. These levels were comparable to that seen previously when CD8+ T cells were adoptively transferred. In contrast, in rats which received CD4+ T cells from donor animals injected with PLA_2 alone, the PLA_2-specific IgE response was not reduced (mean 1585 U/ml) and neither were the total serum IgE levels (mean 3984 ng/ml). Both were significantly greater than preimmunization ($P < 0.001$). Rats receiving CD4+ T cells derived from donor rats immunized with both ricin and antigen had antigen-specific (mean 2652 U/ml) and total IgE levels (mean 6214 ng/ml) comparable to those seen in rats from the other two groups.

Specificity of CD8+ T-suppressor cells

The specificity of the suppression of the IgE response by ricin-sensitive CD8+ T cells was studied by adoptive transfer.

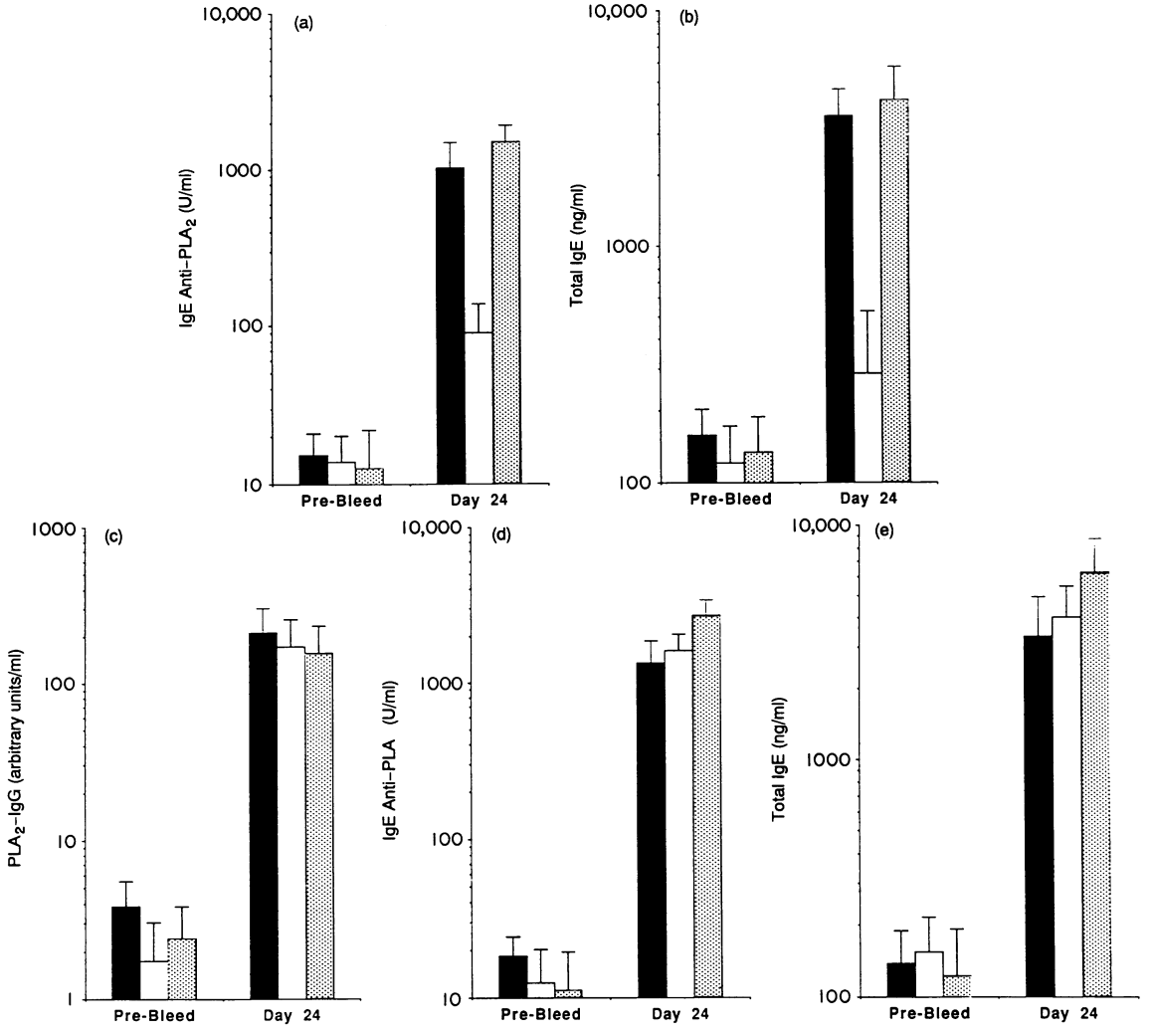

Figure 7. The IgE antibody response to (a) antigen and the total IgE levels (b) in the sera of rats immunized with 10 μg PLA_2 and 100 μg $AlOH_3$, after receiving 1×10^8 $CD8^+$ T cells from donor rats immunized with saline (■), PLA_2 (□) or PLA_2+ricin (▨). The IgG antibody response in the same rats (c). The antigen-specific antibody response (d) and the levels of IgE in the sera (e) of rats which received 1×10^8 $CD4^+$ T cells from donor rats immunized as for (a–c) 24 hr earlier. The data are expressed as the mean and standard deviation.

Adoptive transfer of $CD8^+$ T cells (>98·3% $CD8^+$ and >99·1% $CD5^+$) was performed as before from rats given saline or immunized 3 days previously with PLA_2 alone or PLA_2 and ricin. The recipient animals were then immunized with a different antigen (10 μg OVA) and $Al(OH)_3$. Adoptive transfer of $CD8^+$ T cells from saline-treated donor rats resulted in raised OVA-specific IgE (mean 866 U/ml) (Fig. 8a) and total serum IgE levels (mean 2873 ng/ml) (Fig. 8b) in recipient rats. In rats which received $CD8^+$ T cells from PLA_2-immunized donors, following immunization with OVA and $Al(OH)_3$, there was a marked reduction (66%) in both OVA-specific IgE (mean 206 U/ml) and total serum IgE (76%) levels (mean 989 ng/ml) ($P < 0{\cdot}01$ for both). In recipients of $CD8^+$ T cells from donor rats immunized with both ricin and PLA_2 there was no reduction in either OVA-specific (mean 2014 U/ml) or total IgE levels (mean 2347 ng/ml).

DISCUSSION

Administration of ricin to rats produces a potentiated IgE antibody response which is dependent upon ricin being administered together with or shortly after antigen. This suggests that ricin is acting on a newly activated population of cells. Although it has previously been shown that proliferation of mitogen-stimulated $CD8^+$ T cells is more readily inhibited by ricin than that of comparable $CD4^+$ T cells,[25] no difference was seen in the capacity of the two T-cell subtypes to bind ricin. However, within 24 hr of immunization with 10 μg of antigen there was a substantial increase in the number of ricin-binding sites on $CD8^+$ but not $CD4^+$ T cells recovered from the spleens of the immunized animals. The increased binding of ricin to the surface of activated $CD8^+$ T cells makes it more likely that these cells will bind and internalize injected ricin. Once internalized, the number of ricin molecules needed to kill or inactivate a cell is

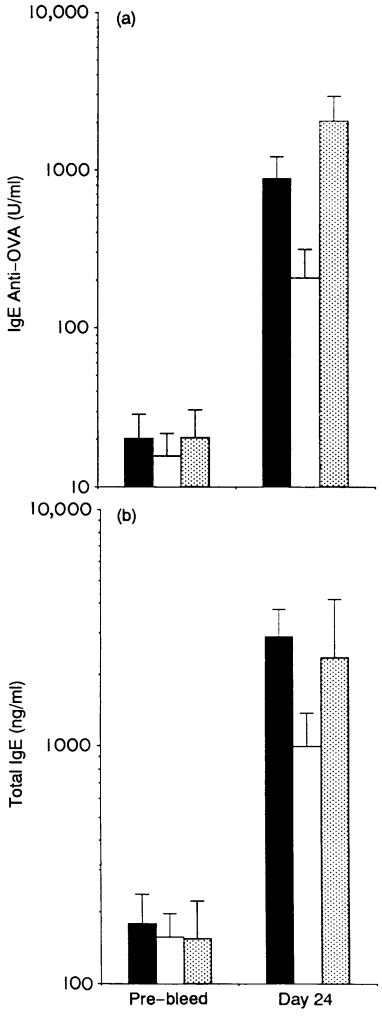

Figure 8. The IgE antibody response to OVA (a) and the levels of total IgE (b) in the sera of rats immunized with 10 μg OVA and 100 μg $AlOH_3$ after receiving 1×10^8 $CD8^+$ T cells from donor rats which had been injected with saline (■), PLA_2 (□) or PLA_2 + ricin (▨) 24 hr before. The data are expressed as the mean and standard deviation.

one; however, *in vivo* the main target of ricin is the hepatocyte.[26] In the spleen there will be insufficient ricin to bind to all the cells present and as a consequence an increase in the number of binding sites on some T cells could explain the decrease in the number of $CD8^+$ T cells in the spleens of rats immunized with ricin and PLA_2. Increased susceptibility to ricin is observed in a large number of $CD8^+$ T cells 24 hr after immunization with PLA_2. The finding that this can also be seen after immunization with OVA (Fig. 6) shows that this is a general feature of immunization and is not due specifically to the nature of the antigen. Although activation is presumably antigen driven, the effects seen are at least in part, antigen non-specific. The observation that some T cells are activated following immunization in an antigen non-specific manner is suprising and to some extent mimics the production of low-affinity IgM antibodies seen early in the immune response. The fact that these cells may reguate IgE is not without precedent; Okumura and Tada[4] showed that anti-thymocyte antiserum given shortly after immunization of rats enhanced IgE production. The same antiserum was ineffective if given before immunization. In addition, although not explored here, it is possible that this non-specific activation may be a result of interaction of $CD8^+$ T cells with accessory cells.

The K_a for ricin binding to T cells obtained in this study agrees with that previously reported using erythrocytes,[37] fibroblasts and monocytes.[27] Ricin is a 60,000 MW protein composed of two subunits, α and β. It binds to the surface of T cells via its two galactose-binding sites present on the 30,000 MW β-subunit; additionally it may bind to monocytes via mannose receptors present on these cells.[27] Addition of lactose, which is a competitive inhibitor for the galactose-binding sites, prevents ricin from binding to both $CD4^+$ and $CD8^+$ T cells. Mannan, which is a competitive inhibitor for the cell surface mannose receptor has no effect on binding to either sub-population.[25] Thus ricin appears to be binding to T cells via its galactose receptors. An increase in the amount of cell surface glycoproteins containing galactose on activated $CD8^+$ but not $CD4^+$ T cells provides a possible explanation for the observation that these cells are more sensitive to ricin *in vivo* following immunization and when stimulated *in vitro* with PHA or Con A.[25] Differential binding of ricin and other lectins has also been reported to mouse thymocytes.[38]

The selective loss of early activated $CD8^+$ T cells and the concomitant enhancement of IgE biosynthesis suggests that ricin potentiates IgE responses by the selective depletion of IgE suppressor cells. The adoptive transfer experiments described here clearly indicate that it is possible to suppress IgE responses with $CD8^+$ T cells, confirming earlier reports by Sedgwick and Holt.[5,14,39] The fact that purified $CD8^+$ T cells derived from rats given both antigen and ricin were unable to suppress IgE supports the hypothesis that ricin inactivates a subpopulation of $CD8^+$ T cells that suppress IgE. The finding that these cells were generated within 3 days of immunization differs from previous reports of $CD8^+$ IgE suppressor T cells[14] which were derived from the spleen and lymph nodes of donor rats which had received six weekly exposures to aerosolized antigen. Furthermore, these cells are reported to be antigen-specific.[40] In this study $CD8^+$ T cells from rats immunized with PLA_2 suppressed the IgE response to a different antigen although this suppression was significantly less than that to PLA_2 itself ($P < 0{\cdot}05$). The fact that IgE production is enhanced if ricin is given together with, or shortly after, antigen but has only a minimal effect if administered before antigen could explain why ricin alone does not enhance IgE poduction.

Although the role of $CD8^+$ T cells in IgE regulation has not previously been studied extensively there is evidence to indicate that these cells may be involved in other forms of immunosuppression. $CD8^+$ T cells have been shown to act as suppressors in autoimmunity[41] and following vaccination.[42] $CD8^+$ suppressor T-cell lines have been produced from animals in whom experimental allergic encephalomyelitis had been induced.[43] $CD8^+$ T cells can act to suppress autoreactive T cells and to inhibit anti-class II $CD8^+$ T cells in normal rats.[44] $CD8^+$ T cells generated in rats following suppression of IgE production by allergen inhalation can suppress IgE production when adoptively transferred to naive recipients.[14,39] These $CD8^+$ T cells were reported to lack the α/β T-cell receptor.[40] Mercuric chloride, which enhances IgE production,[45] inhibits the generation of emergent $CD8^+$ T cells.[44] Low concentrations of cyclosporin A which has

been shown to be an IgE co-stimulator in the mouse,[46,47] preferentially inhibits mitogen-induced proliferation of CD8$^+$ T cells.[48] Other immunosuppressive agents such as sublethal X-ray irradiation,[49] cyclophosphamide[50] and anti-thymocyte globulin[51] also enhance IgE production. In particular, it seems likely that the anti-thymocyte antiserum[51] which was shown by Tada and co-workers to enhance rat IgE responses, only when administered 24 hr after immunization, might have targeted the same early activated CD8$^+$ T-cell subpopulation. Both ricin and the anti-thymocyte antiserum seem to eliminate IgE suppression in an antigen non-specific manner. The fact that ricin elicits a long-lived IgE response, not only in high IgE responder strains of rat such as the Brown Norway, but also in strains such as the Wistar rat[18] suggests that it is active suppression of IgE responses that prevents low IgE responder strains from producing IgE, rather than an inability of these animals to provide appropriate IgE help. It cannot be ruled out that some of the suppressive effects observed are due to antigen presentation and the effect of ricin may not be confined to T cells alone; it is worth noting that inhibitory effects of cyclosporine A may act on both antigen-presenting dendritic cells and on lymphocytes.[52]

We propose that ricin inactivates a subpopulation of CD8$^+$ T cells which normally suppresses IgE production in an antigen non-specific manner. The mechanism by which these cells regulate IgE is as yet unknown; however, recent work has shown that the rise in IgE synthesis induced by ricin and antigen is correlated with a fall in IFN-γ production by splenic T cells.[53] It has further been established that CD8$^+$ T cells are the main source of IFN-γ in the spleen and that the fall in IFN-γ production is most marked in this cell type (manuscript in preparation). IFN-γ is known to inhibit IgE secretion during *in vivo* humoral responses[9] and it is likely that elimination of IFN-γ-producing cells by ricin would result in enhanced IgE production.

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