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Elimination of IgE regulatory rat CD8$^+$ T cells *in vivo* increases the co-ordinate expression of Th2 cytokines IL-4, IL-5 and IL-10

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SUMMARY

Immunization of rats with soluble antigen (ovalbumin) and the castor bean toxin, ricin, eliminates a subpopulation of CD8$^+$ T cells which suppress IgE responses *in vivo*. This treatment also reduces the ability of splenic T cells to produce interferon-γ (IFN-γ) and enhances their capacity to make interleukin-4 (IL-4). In this report we describe the effect of immunization with ricin and antigen on the expression of mRNA for other T-helper type 2 (Th2) cytokines—IL-5 and IL-10—and their relationship to serum IgE and IL-4 mRNA expression. Splenocytes were taken from rats at different times after immunization with antigen or ricin and antigen and activated *in vitro* with phorbol myristate acetate (PMA) and ionomycin for 6 hr and total RNA extracted and reverse transcribed. Cytokine gene expression was detected using a quantitative polymerase chain reaction (PCR). Expression of IL-4, IL-5, and IL-10 was increased 7–20-fold 11 days after immunization with ricin and antigen (from 0·107% to 0·769% β-actin for IL-4, from 0·0167% to 0·381% β-actin for IL-5, and from 0·0581% to 0·954% β-actin for IL-10), and preceded maximum serum IgE levels by 4–5 days. There was no increase in IgE or mRNA for these cytokines in rats immunized with antigen alone. The level of IL-4, IL-5, and IL-10 expression declined rapidly after 12 days. Our results suggest that immunization with antigen and ricin preferentially induces a Th2 response, and that CD8$^+$ T cells may play a part in down-regulating the development of Th2 T cells.

INTRODUCTION

Following immunization, antigen-specific CD4$^+$ T cells are produced. These cells can be broadly classified functionally as T-helper type 1 (Th1) or T-helper type 2 (Th2) cells on the basis of the cytokines that they produce,[1] although other subtypes probably exist too. Th1 and Th2 T cells are believed to arise from naive T cells, which only produce interleukin-2 (IL-2), via intermediate Th0 cells which exhibit an unrestricted cytokine profile. Production of Th2-like cells, which produce IL-4, IL-5, IL-6 and IL-10,[1] has been associated with allergic inflammation[2] and IgE production.[3] The factors which determine whether a Th1 or a Th2 response will result following immunization are still poorly understood. Furthermore, both the nature of the antigen[4] and the antigen-presenting cell[5] appear to influence the generation of a Th1 or Th2 response. The presence of different cytokines during peripheral T-cell development appears to be a major factor in this process and lymphokines such as IL-4 and interferon-γ (IFN-γ) have been shown to regulate Th1 and Th2 development *in vitro* and *in vivo*.[6-8]

We have investigated the induction of Th1/Th2 responses in rats immunized with a soluble antigen (ovalbumin) and the toxic lectin ricin. Ricin, a constituent of castor beans, induces vigorous IgE responses in mice and rats when administered with a soluble antigen, but has no effect on the IgG response.[9] Ricin has been previously shown to eliminate a subpopulation of CD8$^+$ T cells, activated within 24 hr of immunization with antigen, which have increased numbers of ricin-binding sites on their surface. When adoptively transferred into syngeneic recipients, these cells suppress IgE responses to antigen and aluminium hydroxide.[10] We have previously shown that spleen cells from these animals have a decreased capacity to produce IFN-γ and an increased ability to generate IL-4 mRNA.[11] Since IFN-γ is produced by Th1 cells and IL-4 by Th2 cells, this suggested that ricin-sensitive CD8$^+$ T cells might favour the development of a Th1 response. We have therefore investigated

Abbreviations: Con A, concanavalin A; dNTP, deoxynucleotide triphosphate (dNTP); ELISA, enzyme-linked immunosorbent assay; IFN-γ, interferon-γ; IL-2, interleukin-2; IL-4, interleukin-4; IL-5, interleukin-5; PCR, polymerase chain reaction; PMA, phorbol myristate acetate; Th1, T-helper type 1; Th2, T-helper type 2.

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the ability of splenocytes from these animals to express elevated levels of mRNA for other Th2 cytokines.

Spleens from female Hooded Lister rats (Harlon-Olac Ltd, Bicester, U.K.) were taken at different times after intraperitoneal immunization with 10 μg ovalbumin (Sigma Ltd, Poole, U.K.) and 100 ng purified ricin (kind gift from Dr P. Thorpe, ICRF, London, U.K.), or with antigen alone, and splenocytes were extracted as described previously.[11] 3×10^7 cells were cultured in 10 ml complete medium—RPMI adjusted to 310 mOsm + 2·2 g/l sodium carbonate, 0·3 g/l L-glutamine, 110 mg/l sodium pyruvate, 2×10^{-4}% 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum (FCS) (reagents from Gibco Ltd, Paisley, U.K.)—in 50-ml Falcon tubes. Phorbol myristate acetate (PMA; Sigma) was added at 10 ng/ml and ionomycin (Novabiochem, Nottingham, U.K.) at 400 ng/ml. Cells were incubated at 37°, 5% CO_2 in air for 6 hr. RNA was then prepared from the cells using guanidine isothiocyanate lysis.[12] RNA pellets were washed twice with 80% ethanol, air dried and dissolved in 0·5 U/μl RNAguard (Pharmacia, Milton Keynes, U.K.), 5 mM dithiothreitol (BDH Ltd, Dagenham, U.K.). Four micrograms of RNA from each sample were reverse-transcribed as described elsewhere.[13] Quantitative PCR analysis[13] for cytokine mRNAs was then performed using 5 μl reverse-transcribed RNA in 40 μl reverse transcriptase buffer (50 mM Tris-HCl, pH 8·0, 50 mM KCl, 5 mM $MgCl_2$) containing 1·25 μM each primer, 0·3 μl 25 mM dNTPs (Pharmacia), 0·35 U Perfect Match (Stratagene Ltd, Cambridge, U.K.) and 1·2 U Amplitaq polymerase (ILS Ltd, London, U.K.). IL-4 primers were designed from the rat IL-4 cDNA sequence.[14] Their sequences were: IL-4, 5′ACCTTGCTGTCACCCTGTTCTGC3′ and 5′GTTGTGAGCGTGGACTCATTCACG3′, which amplify a 352 base-pair (bp) fragment; IL-5, 5′TGCTTCTGTGCTTGAACGTTCTAAC3′ and 5′TTCTCTTTTTGTCCGTCAATGTATTTC3′, which amplify a 298 bp fragment and were obtained from the rat IL-5 cDNA sequence;[15] IL-10, 5′TGCCAACCCCTTGTCAGAAATGATCAAG3′ and 5′GTATCCAGAGGGTCTTCAGCTTCTCTC3′, which amplify a 127 bp fragment and were derived from a partial cDNA sequence for rat IL-10 (M. Dallman, Nuffield Department of Surgery, John Radcliffe Hospital, Oxford, U.K.); β-actin, 5′AGAAGAGCTATGAGCTGCCTGACG3′ and 5′CTTCTGCATCCTGTCAGCCTACG3′, which produce a 236 bp product and were derived from the rat β-actin sequence.[16] Reaction mixtures were overlaid with mineral oil and up to 29 PCR cycles were performed (94° for 1 min, 60° for 2 min or 15 min for the first four cycles, 72° for 2 min) and aliquots (6 μl) were taken from each sample after two or three different numbers of cycles. For IL-10 PCRs an annealing temperature of 70° was used instead of 60°. Aliquots of reactions were run on 3·2% agarose gels in glycine buffer stained with ethidium bromide. Photographs were taken under UV illumination on Agfa APX25 film and the negatives were scanned on a Molecular Dynamics ImageQuant™ densitometer. Densities of marker fragments (pUC18/*Hpa*II or OX174/*Hin*fI digests) were plotted against molecular weight and used to estimate yields of PCR target fragments. Copies of mRNA per μg total RNA were calculated as described elsewhere.[13] Results were expressed as a percentage of β-actin mRNA copies. Positive controls consisting of plasmid or PCR-generated DNA were run in each assay. Their values varied ±45% between PCR assays. We also observed considerable variation in cytokine expression between different groups of animals (±65%). For this reason experiments were performed using groups of animals which were killed and analysed by PCR simultaneously.

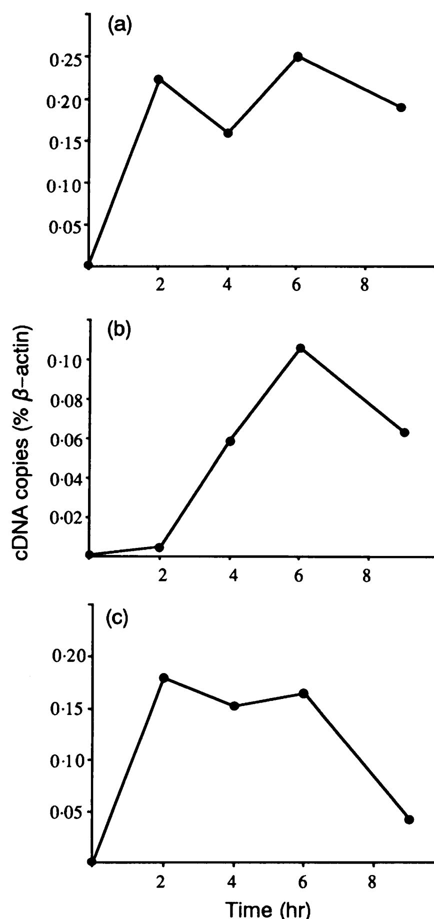

Figure 1. Time-course of expression of IL-4 (a), IL-5 (b) and IL-10 (c) mRNA in normal rat spleen cells after activation with PMA and ionomycin. Cells were incubated for various times in culture medium prior to RNA extraction and quantitative PCR. Results are expressed as a percentage of control β-actin mRNA levels in the same samples. Results are representative of three independent experiments.

The time-course for activation of IL-4, IL-5, and IL-10 genes was determined by PCR using splenocytes from naive rats. Expression of all three Th2 genes was rapidly induced by PMA and ionomycin, reaching a maximum at around 6 hr and then declining (Fig. 1). IL-4 and IL-10 mRNA were detectable after 2 hr and declined after 6 hr stimulation. IL-5 mRNA expression was induced slightly later but was also maximal at 6 hr. It is possible that this difference could be due to IL-4 and IL-10 expression by non-T cells which are more rapidly activated by PMA and ionomycin than T cells. All subsequent assays were carried out 6 hr after stimulation of the cells.

The levels of IL-4, IL-5, and IL-10 mRNA detected in splenocytes from ricin and antigen immunized animals 6 hr after activation, and corresponding serum IgE levels are shown in Fig. 2. Co-ordinate expression of IL-4, IL-5, and IL-10 increased 7–20-fold 11 days after immunization with ricin and antigen—from 0·107% to 0·769% β-actin for IL-4, from 0·0167% to 0·381% β-actin for IL-5, and from 0·0581% to

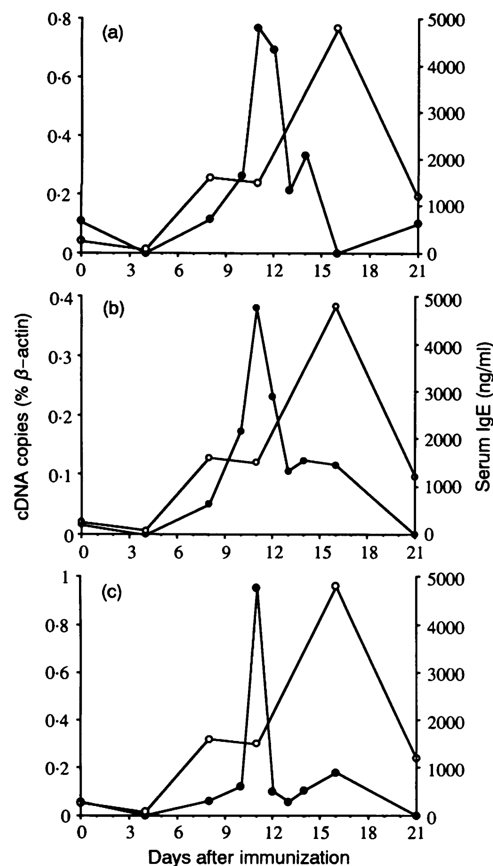

Figure 2. Expression of IL-4 (a), IL-5 (b) and IL-10 (c) mRNA in PMA+ionomycin-stimulated splenocytes from rats at different times after immunization with antigen (ovalbumin) and ricin (●), and serum total IgE levels (○) in the same animals.

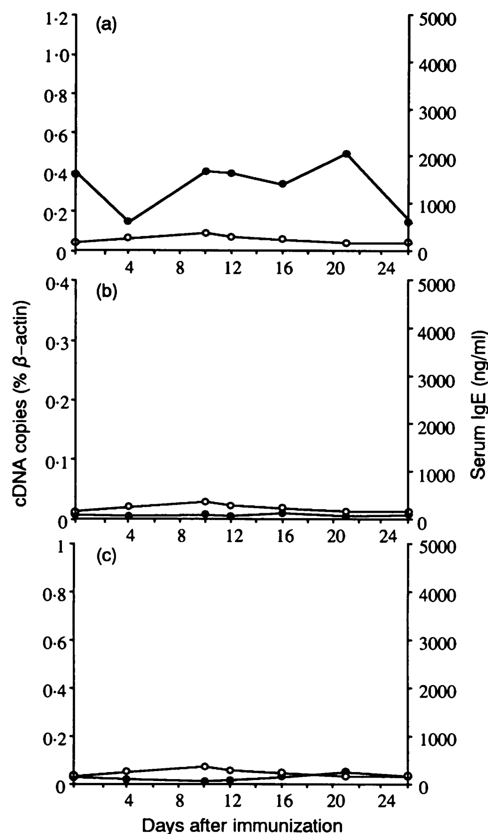

Figure 3. Expression of IL-4 (a), IL-5 (b) and IL-10 (c) mRNA in PMA+ionomycin-stimulated splenocytes from rats at different times after immunization with antigen (ovalbumin) alone (●), and serum total IgE levels (○) in the same animals.

0·954% β-actin for IL-10. This increased capacity for Th2 cytokine expression preceded maximum serum IgE levels by 4–5 days. There was no increase in serum IgE or mRNA for these cytokines in rats immunized with antigen alone (Fig. 3). The increased capacity of splenocytes to express the IL-4, IL-5, and IL-10 genes declined rapidly after 12 days. Since expression of all three cytokines increased at the same time, it seems likely that an expansion of Th2-like cells had occurred. The subsequent rapid decline in cytokine gene expression to control levels may reflect the observation made in the mouse that Th2-like 'effector' cells, which produce high levels of cytokines and provide help for humoral immunity, are short-lived.[17] The rapid increase in serum IgE we observed 4 days after the expansion in Th2 cytokine expression suggests that these Th2 cells are able to induce IgE synthesis in B cells by producing high levels of IL-4. Increased IL-5 production may also support the development of IgE-producing B cells.[18] In addition, simultaneous secretion of IL-10 may contribute to the down-regulation of the T-cell IFN-γ production seen during the response, since IL-10 is reported to suppress Th1 cytokine production.[19] The simultaneous expression of IL-10 with IL-4 and IL-5 in the rat is interesting since the existence of the Th1 and Th2 patterns of cytokine production is unclear in human systems. IL-10 is produced by human Th1 clones and can suppress cytokine production by Th2 as well as Th1 clones.[20] Thus IL-10 may have different roles in humans and rodents.

The enhanced IgE response observed in rats immunized with ricin and antigen appears to be closely related to the ability of splenocytes to produce Th2 cytokines. This differs from studies in mice in which spontaneous cytokine expression was studied during IgE responses induced by injection of anti-IgD.[21] In this case a clear Th2 pattern of cytokine production was not observed. Thus polyclonal stimulation of the immune system, as in the IgD model, may not induce the same pattern of cytokine production which occurs after antigen stimulation. We have previously shown that the ability of ricin to potentiate IgE is due to the enhanced susceptibility of antigen-activated $CD8^+$ T cells to its toxic effects. Adoptive transfer of $CD8^+$, but not $CD4^+$, T cells from rats immunized 3 days previously with antigen alone greatly suppresses IgE in recipients immunized with antigen and aluminium hydroxide.[10] It is possible that these suppressor cells function by regulating the differentiation of $CD4^+$ T cells into the Th1 or Th2 phenotypes, and may thus have an important role in directing immune responses in general.

$CD8^+$ T-cell derived IFN-γ could directly suppress class switching to IgE in B cells.[22] Alternatively, $CD8^+$ T cells may regulate $CD4^+$ T-cell development by producing IFN-γ, or other regulatory cytokines, which suppresses the development of Th2 cells and favours Th1 cell growth.[23] Our observation that immunoregulatory $CD8^+$ T cells are activated early in the immune response (within 24 hr), places them in an ideal position

to fulfil such a role. Whether this is a function of all CD8+ T cells is unclear but the likelihood that there are functionally distinct subpopulations of these cells with different cytokine profiles is suggested by studies on CD8+ T-cell clones.[24] The mechanism by which CD8+ T cells suppress IgE production and possibly modulate Th2 responses, and the possible involvement of accessory cells, has yet to be established.

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