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Interleukin-1β partially alleviates cyclosporin A-induced suppression of IgG1 isotype response to thyroglobulin in BALB/c mice *in vivo*

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SUMMARY

Cyclosporin A (CsA) at 120 mg/kg body weight when injected subcutaneously into BALB/c mice along with thyroglobulin emulsified in incomplete Freund's adjuvant (IFA) was found to suppress antigen-specific IgG titre by 86%. Isotyping revealed that both IgG1 and IgG2a titres were suppressed by 87% and 57%, respectively. But under identical conditions when complete Freund's adjuvant (CFA) was used, the suppression of antigen-specific IgG, IgG1 and IgG2a titres was 50%, 51% and 55%, respectively. Injection of anti-IL-1β-neutralizing hamster monoclonal antibodies along with thyroglobulin and CsA emulsified in CFA increased the suppression of antigen-specific IgG titre. Under such conditions the IgG1 titre was suppressed more than the IgG2a titre. Recombinant human interleukin-1 receptor antagonist (rhuIL-1ra) also enhanced the suppression caused by CsA in the presence of CFA but control hamster immunoglobulin had no such effect. Recombinant human IL-1β, when administered along with thyroglobulin and CsA emulsified in IFA, alleviated the suppression of antigen-specific IgG titre and the IgG1 titre was alleviated more than the IgG2a titre. Under identical conditions, rhuIL-1ra did not alleviate CsA-induced suppression. Lymphocytes from the lymph nodes of thyroglobulin-sensitized BALB/c mice when stimulated *in vitro* by thyroglobulin in the presence of CsA, secreted very little interferon-γ (IFN-γ) and IL-4, but on addition of an optimal dose of rhuIL-1β, IFN-γ and IL-4 secretion was partially restored.

INTRODUCTION

The nature and magnitude of the immune response an antigen elicits *in vivo* after its entry into a host are determined to a great extent by the cytokine milieu in which it encounters the antigen-specific naive $CD4^+$ T cells and B cells. While naive $CD4^+$ T cells undergo priming by their specific antigen to develop into effector cells, they require interleukin-2 (IL-2) as a growth factor,[1] but if other cytokines, such as IL-4 or interferon-γ (IFN-γ), are also present they exert a cross-regulatory effect.[2–6] Therefore, any agent which can alter the cytokine milieu by directly affecting cytokine gene expression when administered along with antigen, is expected to modulate the immune response significantly. Cyclosporin A (CsA) can inhibit both the humoral and the cell-mediated immune response by formation of a CsA–Cyclophilin complex which inhibits the phosphatase activity of calcineurin, thereby affecting dephosphorylation of nuclear factors of activated T cells (NFAT), required for expression of IL-2 and other cytokine genes.[7–11] Therefore, in the presence of CsA, production of IL-2 is inhibited and all IL-2-dependent cell proliferations are blocked. Thus coinjection of CsA along with antigen would affect the nature of antigen-specific effector T-helper cells that would be generated *in vivo*. While a significant amount of information is available about the effects of CsA on the cellular immune response, we found that very limited data are reported about its effect on the nature of different isotype antibodies generated *in vivo* against any antigen. Antigens, when administered emulsified in incomplete Freund's adjuvant (IFA), are much less immunogenic in comparison to when administered emulsified in complete Freund's adjuvant (CFA) because IFA cannot activate antigen-presenting cells effectively and induce secretion of enough cytokines.[12] Therefore, we felt that it would be interesting to study the effect of CsA on the nature of antibody isotypes induced, when antigen is administered along with CsA emulsified in a weak adjuvant like IFA and compare it with the antibodies generated when the antigen is administered along with CsA emulsified in a strong adjuvant like CFA.

We found that coinjection of an immunosuppressive dose of CsA (120 mg/kg body weight) along with thyroglobulin (antigen) emulsified in IFA into BALB/c mice caused suppression of the antigen-specific IgG1 and IgG2a response by 87% and 57%, respectively. Under identical conditions, coinjection of CsA with thyroglobulin emulsified in CFA caused

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Abbreviations: CsA, cyclosporin A; IL-1β, interleukin-1β; IL-1ra, interleukin-1 receptor antagonist; Th1, T-helper-cell type 1; Th2, T-helper-cell type 2.

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suppression of the antigen-specific IgG1 and IgG2a response only by 51% and 55%, respectively. This suppression was not enhanced even when a higher dose of CsA (160 mg/kg body weight) was administered along with antigen emulsified in CFA. Thus when the antigen was emulsified in IFA, CsA affected the IgG1 response much more than when it was emulsified in CFA.

MATERIALS AND METHODS

Mice

Female BALB/c mice, 4–6 weeks old, obtained from the National Institute of Nutrition, Hyderabad, were used in our study.

Cytokines, cytokine kits and anti-IL-1β-neutralizing antibodies

IL-1β and IL-1 receptor antagonist (IL-1ra) were obtained from the National Institute for Biological Standards and Control (NIBSC), Hertfordshire, UK. Monoclonal hamster anti-mouse IL-1β-neutralizing antibody, Intertest-4 and Intertest-γ kits for IL-4 and IFN-γ assays, were obtained from Genzyme (Cambridge, MA).

Determination of immunosuppressive dose of CsA for BALB/c mice

The dose of CsA [Sandimmune, Sandoz (India), Bombay, India] needed for maximal suppression of antibody response in BALB/c mice was determined by subcutaneously injecting different doses of CsA (40–250 mg/kg body weight) along with thyroglobulin (50 μg/mouse) emulsified in IFA or in CFA into different groups of animals on day 0 and day 21 and bleeding them on day 28 and assaying antibody titres. The minimum dose to cause maximum immune suppression was found to be 120 mg/kg body weight of CsA. This dose was used as the experimental dose of CsA.

Antigen and immunization

BALB/c mice (10 in each group) were immunized subcutaneously with 50 μg thyroglobulin (Sigma, St Louis, MO) per mouse, emulsified either in IFA (group-1) or in CFA (group-2) as control groups. The third and fourth groups of mice were immunized as for groups 1 and 2 but with an experimental dose of CsA. The fifth and sixth groups of mice were immunized with antigen emulsified in IFA and an optimal dose of human IL-1β (NIBSC; 1000 IU/mouse), without or with CsA. The seventh and eighth groups of mice were immunized with antigen emulsified in IFA and an optimal dose of IL-1ra (NIBSC; 20 IU/mouse), without or with CsA. The ninth and tenth groups of mice were immunized with antigen emulsified in CFA in the presence of an optimal dose of hamster anti-IL-1β-neutralizing antibody (Genzyme; 15 μg/mouse – at 0 hr, 24 hr, 48 hr), without or with CsA. The eleventh group of mice were immunized with antigen emulsified in CFA containing CsA and normal hamster immunoglobulin (15 μg/mouse) as control. The twelfth group of mice were immunized with antigen emulsified in CFA with IL-1ra (20 IU/mouse). The thirteenth group of mice were immunized with antigen emulsified in CFA with CsA and IL-1ra. Optimal doses for IL-1β, IL-1ra and anti-IL-1β neutralizing antybody were determined by separate experiments. A booster dose, as per the previous immunization protocol, was given to all the respective groups (groups 1–13) on day 21 and blood was collected from individual mice from each group on day 28. Sera were then prepared and stored at −70°.

Enzyme-linked immunosorbent assay (ELISA)

Thyroglobulin (1 μg in 100 μl of 0·05 M carbonate/bicarbonate buffer, pH 9·5 per well) was coated to ELISA plates (Nunc, Roskilde, Denmark), then blocked with 1% bovine serum albumin (BSA) in the same buffer. ELISA was performed by using serially diluted antisera from different groups. Isotyping of antibodies was done following the published protocol.[13]

In vitro *stimulation of lymph node cells from sensitized BALB/c mice*

Lymph node cells (2×10^5 cells in 200 μl of medium/well) from thyroglobulin-sensitized BALB/c mice (11 days after sensitization) were cultured in medium [RPMI-1640, 10% fetal calf serum (FCS) with optimal concentration of thyroglobulin (10 μg/ml)] and with optimal suppressive dose of CsA (100 ng/ml) or optimal concentration of human IL-1β, (100 IU/ml), or optimal concentration of IL-1ra, (10 IU/ml) at 37° in a humid chamber containing 5% CO_2. All the optimal doses were first determined by separate titrations. Supernatants were taken after 24 hr and 36 hr, two early time-points at which a maximal amount of IL-4 and IFN-γ, respectively, were found to be secreted and stored at −70° for quantification. For measuring lymphocyte proliferation, lymph node cells were cultured for 48 hr with or without CsA and pulsed with [^{3}H]thymidine (1 μCi/well) for 24 hr. Cells were washed and then lysed with 1% sodium dodecyl sulphate (SDS) and put in Cocktail-T. Counts per minute (c.p.m.) was read in a β-scintillation counter.

Sandwich ELISA for cytokine assay

To quantify IL-4 and IFN-γ in culture supernatants, Genzyme Intertest-4 and Intertest-γ kits, respectively, were used. Briefly, capture antibodies (hamster monoclonal anti-IL-4 or anti-IFN-γ) at 2 μg/ml in 0·1 M carbonate/bicarbonate buffer, pH 9·6 were coated to ELISA plates (Nunc) and culture supernatants along with cytokine standards were used to quantify IL-4 and IFN-γ by sandwich ELISA.

Macrophage culture and IL-1β assay

BALB/c mice were injected intraperitoneally with IFA, CFA, or phosphate-buffered saline (PBS) (250 μl/mouse), respectively. They were killed after 72 hr by cervical dislocation and peritoneal macrophages were isolated and cultured separately at 37° in a 5% CO_2 atmosphere in 24-well flat-bottom plates at 1×10^6 cells/well in RPMI-1640 medium with 5% FCS following a published procedure.[14] Culture supernatants were collected after 24 hr and IL-1β was assayed by [^{3}H]thymidine incorporation using the IL-1β-dependent D10.G4.1 cell line.[15] Briefly, serial dilutions of recombinant standard IL-1β (Sigma) and culture supernatants were incubated with 1×10^4 D10.G4.1 cells in 96-well plates for 72 hr. Pulsed with [^{3}H]thymidine (1 μCi/well) for 24 hr. Mean c.p.m. values from triplicate cultures were converted into pg/ml by fitting values with the standard curve obtained using recombinant IL-1β. Similarly, the effect of a suppressive dose of CsA on the ability of macrophages activated by CFA or IFA to secrete IL-1β was measured by injecting into the peritoneum of another group

of BALB/c mice an immunosuppressive dose of CsA (120 mg/kg body weight) at 0 hr along with CFA or IFA and then CsA alone at 48 hr. Collecting the peritoneal macrophages at 72 hr, culturing them as described above and quantifying secreted IL-1β.

RESULTS

Differential suppression of thyroglobulin-specific antibody response by CsA when coinjected with antigen emulsified in IFA or CFA

The antigen-specific IgG end-point titres after two immunizations were $22 \pm 2 \times 10^3$ and $36 \pm 3 \times 10^3$ in group 1 and 2 mice, respectively (Fig. 1A, a and b). Under identical conditions when an immunosuppressive dose of CsA (120 mg/kg body weight) (Table 1) was injected, the antigen-specific IgG end-point titres were suppressed to $3 \pm 0{\cdot}5 \times 10^3$ and $18 \pm 2 \times 10^3$ in group 3 and group 4, respectively (Fig. 1A, c and d). In the presence of IFA, CsA induced 86% suppression of antigen-specific IgG titre, but in the presence of CFA the suppression was only 50%.

CsA in the presence of IFA suppressed the antigen-specific IgG1 response by 87%, but when CFA was used there was only 51% suppression (Fig. 1B, c and d). The antigen-specific IgG2a isotype titre was reduced by 57% when IFA was used as compared to 55% when CFA was used (Fig. 1C, c and d). Thus CsA caused a greater percentage suppression of antigen-specific IgG1 isotype response in the presence of IFA than in the presence of CFA. But the percentage of suppression of antigen-specific IgG2a titres induced by CsA in the presence of IFA or CFA were not very much different.

Enhanced secretion of IL-1β by peritoneal macrophages activated by CFA compared with that activated by IFA and effect of CsA on IL-1β secretion

When the peritoneal macrophages activated *in vivo* by CFA or IFA were cultured *in vitro* for the secretion of IL-1β, the cells activated by CFA were found to secrete a higher quantity of IL-1β (1500 pg/ml) than those activated by IFA (580 pg/ml) (Table 2). In the presence of CsA, secretion of IL-1β by the macrophages activated by CFA was reduced from 1500 pg/ml to 1400 pg/ml, whereas under identical conditions the same was reduced more drastically from 580 pg/ml to 350 pg/ml in the case of macrophages activated by IFA (Table 2).

Table 1. Suppression of antigen-specific total IgG response to thyroglobulin (Ag) *in vivo* by injection of different doses of CsA emulsified in IFA or CFA. Results are arithmetic mean of anti-thyroglobulin IgG titres of 10 mice ± SD, giving data of one representative experiment of two conducted

CsA (mg/kg body weight)	End-point antibody titre ± SD × 10⁻³ Ag in IFA	Ag in CFA
0	(20·0 ± 3·0)	(35·0 ± 2·5)
40	(15·0 ± 2·0)	(30·0 ± 3·0)
80	(10·0 ± 2·5)	(26·0 ± 2·0)
120*	(3·0 ± 1·0)	(20·0 ± 3·0)
160	(2·8 ± 1·5)	(19·0 ± 4·0)
200	(2·7 ± 0·5)	(20·0 ± 2·0)
250	(2·8 ± 0·6)	(18·0 ± 3·0)

*120 mg/kg body weight of CsA was determined to be the optimal dose for suppression of the humoral response to thyroglobulin.

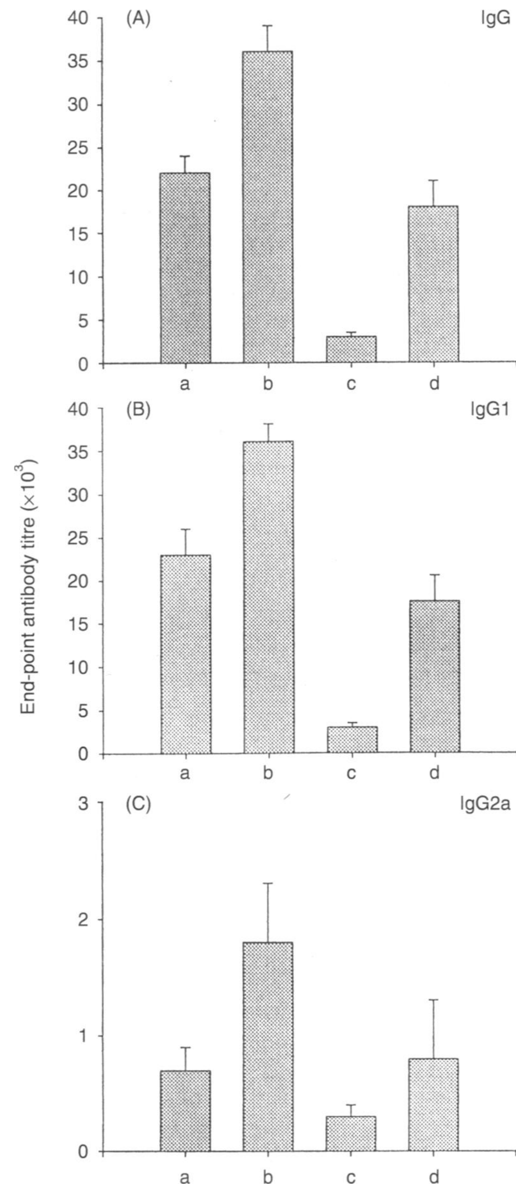

Figure 1. End-point titres of antigen-specific total IgG (A), IgG1 (B) and IgG2a (C) isotype response of BALB/c mice to thyroglobulin antigen after two immunizations as measured by ELISA. Results are arithmetic mean of antibody titres of 10 mice ± SD, giving data of one representative experiment out of three conducted. CsA dose used was 120 mg/kg body weight. a, antigen in IFA; b, antigen in CFA; c, antigen + CsA in IFA; d, antigen + CsA in CFA.

Enhancement of CsA-induced suppression of antibody response to thyroglobulin in the presence of CFA by anti-IL-1β-neutralizing antibody and IL-1ra

The suppression of antigen-specific IgG response to thyroglobulin by CsA in the presence of CFA as adjuvant was further enhanced from 50% to 86% by injecting hamster anti-IL-1β neutralizing monoclonal antibodies (15 μg/mouse) at 0 hr,

Table 2. Secretion of IL-1β by peritoneal macrophages activated *in vivo* by PBS, IFA, CFA, or CFA/IFA in the presence of CsA

Activating agent(s)	IL-1β (pg/ml)
PBS	184±15
IFA	580±45
IFA+CsA (0 hr & 48 hr)	350±25
CFA	1500±100
CFA+CsA (0 hr & 48 hr)	1400±50

Results are arithmetic mean values of triplicate wells±SD, giving data of one representative experiment of three conducted.

D10 cell lines used in this IL-1β assay were cultured *in vitro* for 72 hr followed by 24-hr pulse with [^{3}H]thymidine.

24 hr and 48 hr into group 10 mice (Fig. 2A, d and e). Injection of normal hamster immunoglobulin into group 11 mice under identical conditions did not enhance suppression (Fig. 2A, d and f). When IL-1ra (20 IU/mouse) was injected, suppression increased from 50% to 93% in group 13 mice (Fig. 2A, d and g). Injection of anti-IL1β antibodies or IL-1ra in CFA also induced suppression of antigen-specific IgG response by 36% and 33% in group 9 and group 12 mice, respectively (Fig. 2A, b and c).

Anti-IL-1β-neutralizing hamster monoclonal antibodies enhanced the suppression of IgG1 response from 51% to 85% (Fig. 2B, d and e) but normal hamster immunoglobulin had no such effect (Fig. 2B, d and f) and IL-1ra showed enhancement of suppression from 51% to 93% (Fig. 2B, d and g). The antigen-specific IgG1 isotype responses were also suppressed by 30% and 28%, respectively, when anti-IL-1β antibodies or IL-1ra alone were injected with CFA (Fig. 2B, b and c). The antigen-specific IgG2a response was further suppressed as was the IgG1 response by IL-1β-neutralizing antibodies from 55% to 67% (Fig. 2C, d and e) and by IL-1ra from 55% to 95% (Fig. 2C, d and g). But normal hamster immunoglobulin did not enhance suppression (Fig. 2C, d and f). The antigen-specific IgG2a isotype responses were also suppressed by 44% and 33%, respectively, when anti-IL-1β antibodies or IL-1ra alone were injected along with CFA (Fig. 2C, b and c).

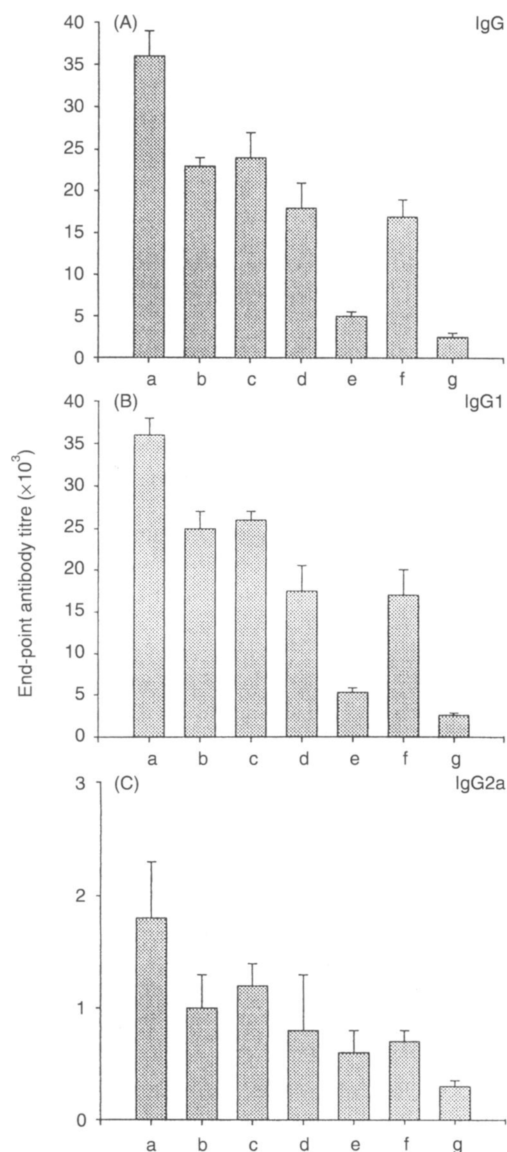

Figure 2. End-point titres of antigen-specific total IgG (A), IgG1 (B) and IgG2a (C) isotype response of BALB/c mice to thyroglobulin antigen after two immunizations as measured by ELISA. Results are arithmetic mean of antibody titres of 10 mice±SD, giving data of one representative experiment out of four conducted. CsA dose used was 120 mg/kg body weight. a, antigen in CFA; b, antigen in CFA with anti-IL-1β-neutralizing antibodies; c, antigen in CFA with IL-1 receptor antagonist; d, antigen+CsA in CFA; e, antigen+CsA in CFA with anti-IL-1β-neutralizing antibodies; f, antigen+CsA in CFA with normal hamster immunoglobulin; g, antigen+CsA in CFA with IL-1 receptor antagonist.

Partial alleviation of CsA-induced suppression of antibody response to thyroglobulin by IL-1β

The suppression of antigen-specific IgG response to thyroglobulin by CsA in the presence of IFA was alleviated by 42% by injecting an optimal dose of recombinant IL-1β (1000 IU/mouse) with the antigen into group 6 mice (Fig. 3A, d and e). But under identical conditions, IL-1ra (20 IU/mouse) alleviated suppression only by 5% in group 8 mice (Fig. 3A, d and f). Injection of IL-1β alone with IFA increased the antigen-specific IgG response by 48% in group 5 mice (Fig. 3A, a and b), while IL-1ra alone injected under the same conditions into group 7 mice suppressed the antigen-specific IgG response by 9% (Fig. 3A, a and c). Analysis of the antigen-specific IgG1 isotype response showed that IL-1β alleviated the suppression of the IgG1 response by 35% (Fig. 3B, d and e) whereas IL-1ra alleviated the response by only 3% (Fig. 3B, d and f). The antigen-specific IgG1 isotype response was enhanced by 39% when IL-1β alone was injected with IFA but injection of IL-1ra alone under similar conditions suppressed the response by 4% (Fig. 3B, a and b, and a and c). The antigen-specific IgG2a suppression was alleviated by 13% when mice were injected with IL-1β along with CsA in IFA (Fig. 3C, d and f) but injection of IL-1ra increased the suppression from 57% to 71% (Fig. 3C, d and f). The antigen-specific IgG2a response was enhanced by 100% when IL-1β alone was injected along with IFA but injection of IL-1ra alone suppressed the response by 14% (Fig. 3C, a and b, and a and c).

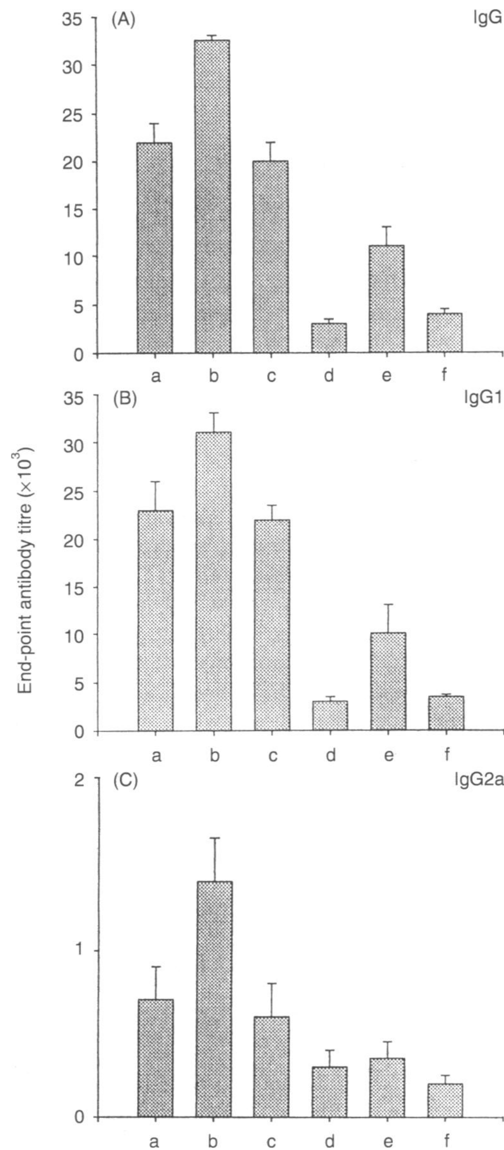

Figure 3. End-point titres of antigen-specific total IgG (A), IgG1 (B) and IgG2a (C) isotype response of BALB/c mice to thyroglobulin antigen after two immunizations as measured by ELISA. Results are arithmetic mean of antibody titres of 10 mice ± SD, giving data of one representative experiment out of three conducted. CsA dose used was 120 mg/kg body weight. a, antigen in IFA; b, antigen in IFA with IL-1β; c, antigen in IFA with IL-1 receptor antagonist; d, antigen + CsA in IFA; e, antigen + CsA in IFA with IL-1β; f, antigen + CsA in IFA with IL-1 receptor antagonist.

Alleviation of CsA-induced suppression of secretion of IL-4 and IFN-γ by IL-1β in thyroglobulin-primed lymphocyte cultures

When lymphocytes from lymph node cells of thyroglobulin-sensitized BALB/c mice were cultured in the presence of an optimal dose of thyroglobulin (10 μg/ml) IL-4 and IFN-γ were secreted at 25 pg/ml (Fig. 4A, a) and 5500 pg/ml, respectively (Fig. 4B, a). In the presence of an optimal dose of CsA (100 ng/ml) (Table 3) the secretion of IL-4 and IFN-γ were reduced by 50% (Fig. 4A, a and d) and 71% (Fig. 4B, a and d), respectively. When the cells were cultured in the presence of antigen, CsA, and optimal dose of IL-1β (100 IU/ml)

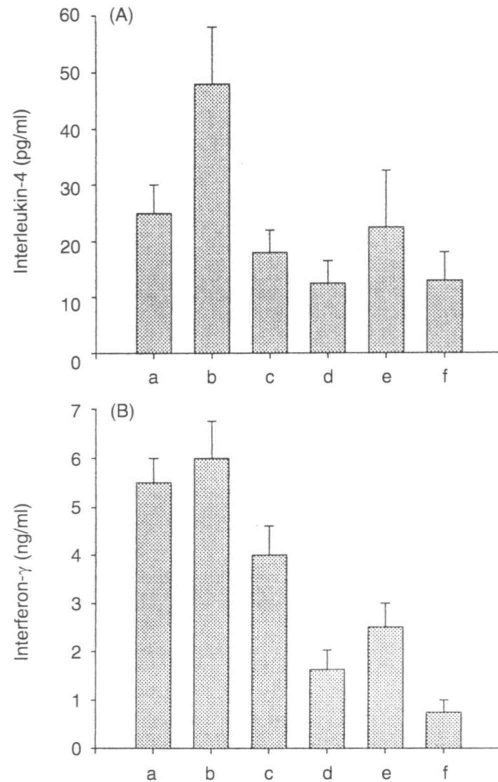

Figure 4. *In vitro* secretion of IL-4 (A) and IFN-γ (B) by the lymphocytes from thyroglobulin-sensitized BALB/c mice stimulated with thyroglobulin (Ag). Results are arithmetic mean values of triplicate wells ± SD, giving data of one representative experiment of three conducted. CsA dose used was 100 ng/ml. a, Ag only; b, Ag + IL-1β; c, Ag + IL-1 receptor antagonist; d, Ag + CsA; e, Ag + CsA + IL-1β; f, Ag + CsA + IL-1 receptor antagonist.

Table 3. *In vitro* suppression of T-cell proliferation by different doses of CsA

Culture condition	c.p.m. ± SD × 10^{-3}	% Suppression
Medium only	(1·50 ± 0·05)	
Ag only	(10·09 ± 1·00)	
Ag + CsA (1)	(7·45 ± 0·50)	30·0
Ag + CsA (25)	(5·75 ± 0·20)	50·0
Ag + CsA (50)	(4·05 ± 0·25)	70·0
Ag + CsA (100)*	(1·93 ± 0·35)	95·0
Ag + CsA (150)	(2·35 ± 0·10)	90·0
Ag + CsA (200)	(2·18 ± 0·20)	92·0
Ag + CsA (400)	(2·01 ± 0·50)	94·0
Ag + CsA (500)	(2·02 ± 0·15)	94·5

Results are arithmetic mean c.p.m. values of triplicate wells ± SD, giving data of one representative experiment of three conducted. CsA used in the range of 1 ng/ml to 500 ng/ml.

Lymph node cells from BALB/c mice sensitized with thyroglobulin (Ag) were cultured *in vitro* for 48 hr followed by 24 hr pulse with [³H]thymidine.

*100 ng/ml was determined to be the optimal dose CsA for the suppression of T-cell response *in vitro*.

Table 4. Alleviation of CsA-induced suppression of T-cell proliferation in the presence of different concentrations of IL-1β

Culture condition	c.p.m. ± SD × 10^{-3}
Medium only	(1·00 ± 0·10)
Ag only	(9·00 ± 0·92)
Ag + CsA (100 ng/ml)	(1·80 ± 0·20)
Ag + CsA + IL-1β (10)	(3·01 ± 0·50)
Ag + CsA + IL-1β (20)	(4·00 ± 0·30)
Ag + CsA + IL-1β (50)	(7·07 ± 0·20)
Ag + CsA + IL-1β (100)*	(8·00 ± 0·50)
Ag + CsA + IL-1β (250)	(6·10 ± 0·25)
Ag + CsA + IL-1β (1000)	(5·01 ± 0·40)

Results are arithmetic mean c.p.m. values of triplicate wells ± SD, giving data of one representative experiment of three conducted. IL-1β used in the range of 10 IU/ml to 1000 IU/ml.

Lymph node cells from BALB/c mice sensitized with thyroglobulin (Ag) were cultured *in vitro* for 48 hr followed by 24 hr pulse with [^{3}H]thymidine.

*100 IU/ml was determined as the optimal dose of IL-1β for alleviation of CsA-induced suppression of T-cell response *in vitro*.

(Table 4) the suppression of IL-4 and IFN-γ were alleviated by 80% and 29%, respectively (Fig. 4A, d and e; Fig. 4B, d and e). Under identical conditions when IL-1ra (10 IU/ml) was used in place of IL-1β the suppression of IL-4 secretion was alleviated only by 4% and no alleviation was observed in the secretion of IFN-γ (Fig. 4A, d and f; Fig. 4B, d and f). Addition of IL-1β to cultures in the presence of thyroglobulin enhanced IL-4 and IFN-γ secretion by 92% and 9%, respectively (Fig. 4A, a and b; Fig. 4B, a and b). But IL-1ra under similar conditions did not have any enhancing effect (Fig. 4A, a and c; Fig. 4B, a and c).

DISCUSSION

When naive CD4$^+$ T (pTh) cells get activated by antigens, they produce IL-2 as their first and major lymphokine.[16] Upon subsequent antigen priming, these cells differentiate into either Th1 effector (eTh1) or eTh2 cells secreting primarily IFN-γ or IL-4, respectively.[2,6] In this process, IL-2 has been shown to be not only needed as a growth factor but also as a differentiation factor for acquisition of IL-4-secreting ability by eTh2 cells.[2,16,17] Adjuvants create an immunogenic milieu for the antigens emulsified in them by activating antigen-presenting cells and generating cytokines.[18,19] Therefore, CFA, which contains killed mycobacteria, is a better adjuvant than IFA.[12] Since the potency of CFA as an adjuvant is related to its ability to induce better cytokine secretion, CsA which inhibits transcription of IL-2 and other cytokine genes is expected to suppress the cytokine-mediated adjuvant effect of CFA more effectively than that of less potent adjuvants like IFA. We have studied the effect of coadministration of CsA (120 mg/kg body weight) on the immune response of BALB/c mice against thyroglobulin emulsified in CFA or IFA. We found that CsA in the presence of CFA was much less immunosuppressive than in the presence of IFA. The thyroglobulin-specific total immunoglobulin (IgG) titre was reduced by 86% compared to the control, when antigen along with CsA was administered emulsified in IFA. But when CFA was used, under identical conditions, the reduction was only 50% (Fig. 1A). Concentrations of CsA higher than 120 mg/kg body weight did not increase its immunosuppressive effect (Table 1). Analysis of the antigen-specific IgG1 and IgG2a titres (Fig. 1B and C) revealed that while the IgG2a titres were reduced to the same extent in both cases, the IgG1 titres were much more suppressed by CsA in the presence of IFA than in the presence of CFA.

Production of IgG1 isotype in mouse is influenced by Th2 cells.[20] It has been reported that some Th2 cell clones require IL-1β to proliferate in the presence of IL-4.[21,22] It is quite likely that CFA induces secretion of a much higher quantity of IL-1β by activating macrophages.[19] This has been demonstrated by us to be so by taking peritoneal macrophages of BALB/c mice activated by IFA and CFA separately and measuring the quantity of IL-1β secreted by equal number of cells over a 24-hr culture period by using the D10 cell proliferation assay (Table 2). If IL-1β is responsible for reduced immunosuppression by CsA in the presence of CFA then neutralization of IL-1β by anti-IL-1β antibodies or blocking its effect by using IL-1ra should increase the observed suppression.

We find that optimal doses of anti-IL-1β-neutralizing hamster antibodies or IL-1r antagonist but not normal hamster immunoglobulin increased the suppressive effect of CsA in the presence of CFA *in vivo*. The suppression of the IgG response was increased to the extent of 86% and 93%, respectively, of the control in comparison to 50% when antigen was administered emulsified in CFA in the presence of CsA (Fig. 2A). The effect on IgG1 isotype response was also equally drastic (Fig. 2B). The anti-IL-1β-neutralizing antibodies and IL-1ra when injected alone had also a suppressive effect on antigen-specific total IgG and IgG1 response (Fig. 2A, b and c; Fig. 2B, b and c).

In the presence of CsA, while synthesis of IL-2 and other cytokines would be reduced, IL-1β production by macrophages will not be affected to that extent.[23,24] We have demonstrated this by culturing peritoneal macrophages of BALB/c mice activated by CFA in the presence of suppressive doses of CsA and quantifying the amount of secreted IL-1β by D10 cell proliferation assay (Table 2). The IL-1β thus produced by macrophages even in the presence of CsA would induce proliferation of certain Th2-type cells which would secrete IL-4 that would help in the production of IgG1 isotype antibodies. By injecting IL-1β-neutralizing antibodies or IL-1r antagonist it is possible to reduce the effect of IL-1β and therefore, under such conditions we observed enhanced suppression. The observed immunosuppression by CsA in the presence of IFA must therefore be partly caused by the inability of IFA to activate macrophages effectively to secrete IL-1β in sufficient quantity. Therefore, if one would administer an optimal dose of IL-1β along with antigen emulsified in IFA in the presence of CsA, there will be less suppression. We find that when an optimal dose of IL-1β was injected along with antigen emulsified in IFA and CsA there was 42% alleviation of suppression of the IgG response. Under similar conditions, when IL-1ra was used as a control, it did not show much alleviation (Fig. 3A). The effect of exogenously added IL-1β on alleviation of IgG1 response was 35% and IL-1ra had very little effect (Fig. 3B). The effect of addition of IL-1β on IgG2a response was very marginal (Fig. 3C). Addition of IL-1β to thyroglobulin emulsified in IFA increased its immunogenicity as evidenced by increased antigen-specific total IgG and IgG1 response. But under similar conditions IL-1ra did not show

any enhancing effect (Fig. 3A, b and c; Fig. 3B, b and c). These observations support our conclusion that the differential immunosuppression by CsA in the presence of IFA and CFA is due to production of a higher quantity of IL-1β by activated macrophages in the presence of CFA. We have demonstrated that peritoneal macrophages activated by CFA produce higher amounts of IL-1β than the ones activated by IFA (Table 2). If IL-1β is capable of inducing proliferation of Th2 cells which secrete IL-4 even in the presence of CsA, it should be possible to test it *in vitro*. By using different concentrations of CsA in thyroglobulin-induced proliferation of primed lymph node-derived T cells, we saw that 100 ng/ml of CsA caused 95% suppression (Table 3). Then under this condition when increasing amounts of rIL-1β were added to the culture it was found that 100 IU/ml of it could bring 86% alleviation of T-cell suppression (Table 4). We found that lymph node cells taken from thyroglobulin-primed BALB/c mice when stimulated with thyroglobulin *in vitro* in the presence of an optimal concentration of CsA produced very little IL-4 and IFN-γ. The optimal dose of IL-1β when added to these cultures alleviated the ability of lymph node cells to produce IL-4 and IFN-γ. But the alleviation of IL-4 production was 80% whereas that of IFN-γ was only 29% (Fig. 4 A and B). Thus, IL-1β is capable of inducing proliferation of Th2 cells even in the presence of CsA. Earlier work had shown that macrophages parasitized *in vitro* with *Leishmania major* can induce proliferation of Th2 cells through release of IL-1[15] and Th2 cells can proliferate in the presence of CsA.[25,26] There are reports that IL-2 and IFN-γ production by Th1 cells is more sensitive to inhibition by Cholera toxin, 8-BR-cAMP and CsA than IL-4 production by Th2 cells.[16,27] Thus IL-1β in the presence of CsA alleviates IL-4 secretion (Th2 cell product) much better than IFN-γ secretion (Th1 cell product). Therefore IL-1β in the presence of thyroglobulin can stimulate proliferation of thyroglobulin-specific Th2 cells in the presence of CsA and this is reflected in the partial alleviation of antigen-specific IgG1 isotype response *in vivo*.

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