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Effect of 17β-Estradiol on Immunoglobulin Secretion by Human Tonsillar Lymphocytes *In Vitro*

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Sex steroid hormones play a role in the complex network of immune responses but the mechanism of their action is still unclear. Effects of a wide range of doses of 17β-estradiol (E2: 0.2–100 ng/ml) on human tonsillar lymphocyte cultures were examined. B and T lymphocyte enriched preparations were stimulated with various concentrations of interleukin-2 and the production of immunoglobulin was measured. Addition of E_2 increased B cell immunoglobulin production in a T cell dependent way with intact T cells being obligatory. The effects of E_2 were also examined on DNA synthesis by tonsillar T cells. E_2 alone caused a significant increase in T cell DNA synthesis. With phytohaemagglutinin-stimulated T cell cultures there was a significant increase in DNA synthesis with E_2 at pharmacological doses. Different cell surface and activation markers (including CD25, p75, HLA-DR, CD28) on tonsillar lymphocytes were also studied after exposure to E_2. The presence of E_2 made no significant difference in the expression of the markers either alone or when the activation antigens were induced by other stimuli. We have shown that intact T cells are needed for the action of E_2 on tonsillar B lymphocyte differentiation and have excluded several mechanisms of action of E_2 since common activation antigens are unaffected.

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INTRODUCTION

Since the last century, evidence has slowly accumulated that sex steroid hormones affect the immune system [1–3]. For example, a sexual dimorphism exists in the immune response with females having higher immunoglobulin levels than males and raising higher antibody titres to viruses and parasites. By contrast, cell-mediated immunity in females is decreased compared to males. Women are more prone to allergy and to autoimmune diseases. Further evidence is that during pregnancy, gonadectomy and sex steroid hormone replacement, the immune response is altered [1–3]. Receptors for estradiol (E_2) have been identified in human peripheral blood mononuclear cells, especially in the $CD8^+$ T cells, thymic cells and splenic lymphocytes [4–6].

There are two hypotheses about the action of E_2 on lymphocytes. First, E_2 may affect lymphocytes indirectly *in vivo* through the thymus by a mechanism which may involve thymic factors [1, 7, 8]. Second, there is evidence that E_2 has a direct effect on lymphocytes in culture [9–16].

In the present study, we have quantified the direct effect of E_2 on human tonsillar lymphocytes *in vitro* principally by measuring immunoglobulin production. To help to elucidate the mechanisms of the action of E_2, we have also studied its effect on T cell proliferation and the expression of lymphocyte activation markers.

MATERIALS AND METHODS

Cell preparations

Human tonsils were obtained after routine operations for chronic tonsillitis and used as sources of B and T lymphocytes [17]. In brief, after the tonsils were teased into medium [RPMI-1640 + 10% foetal calf serum (FCS)], the cell suspension was passed through a fine cell strainer to remove remnants of tissue and then layered onto Ficoll-Paque (Pharmacia, UK). The mononuclear cells obtained from the interface of the Ficoll-Paque were rosetted overnight with neuraminidase-treated sheep erythrocytes (n-SRBC) to separate T cells from a non-T preparation. The T cells were recovered by lysing the treated sheep erythrocytes with ammonium chloride lysis fluid. By flow cytometry, the T cell preparation contained more than 90%

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$CD3^+$ cells and less than 3% $CD19^+$ cells. The non-T preparation was layered onto a Percoll (Sigma, UK) gradient and only the band of high-density cells (45 to 55% Percoll) was used for the B cell preparations. By flow cytometry, there were about 90–95% $CD19^+$ cells and no more than 2% of $CD3^+$ cells.

Cell cultures

B cells (40,000/well) were cultured with or without T cells (80,000/well) in 20 μl hanging drops in 60-well Terasaki plates (Cel-Cult, Sterilin, UK) in 5% CO_2 in air [18]. The medium was RPMI-1640, with penicillin/streptomycin (100 IU/ml) and L-glutamine (2 mM) with heat-inactivated FCS (10%). The FCS used had been screened for E_2 levels which were very low (max. 0.02 ng/ml) compared to the levels added later in cultures (2 to 100 ng/ml). No attempt was made to strip the steroid hormones from the FCS as cultures with and without added E_2 would both be exposed to the same medium and the effects of added E_2 that we report would be unaffected by any background levels of E_2. In pilot experiments we found that the effects of extrinsic E_2 in the dose range that we used was unchanged whether or not the FCS had been stripped of steroid hormones. The minimum number of replicate wells for each set of conditions was six. Initial cell viability was checked with Trypan blue dye exclusion and always exceeded 85%. Cultures were done from 4 different tonsils.

Stimulants

Recombinant interleukin-2 (rIL-2, British Biotechnology Products Ltd, UK) was reconstituted according to the manufacturer's instructions, aliquoted and stored at −70°C. Dilutions in medium were made immediately before use at a range of final concentrations of 10–200 U/ml. E_2 (Sigma) supplied as a lyophilized and gamma-ray irradiated powder, was reconstituted in 1 ml of sterile absolute alcohol followed by 49 ml of RPMI making a stock solution of 20 μg/ml and aliquoted before storage at −70°C. Dilutions of E_2 were made with medium immediately before use at a final concentration ranging from 0.2–100 ng/ml. Phytohaemagglutinin (PHA, Wellcome, UK) was obtained in freeze-dried form, reconstituted in sterile water, stored at −70°C and used at final concentrations of 0.5 and 1 μg/ml.

Immunoglobulin (IgM and IgG) assay

B cell preparations were cultured with or without T cells in the presence of rIL-2 and with or without E_2 for 8 days. In some instances, T cell supernatants replaced intact T cells. To prepare these supernatants, T cells were incubated overnight with IL-2. The supernatant was then added to B cells and the cultures stimulated in the presence of IL-2 and E_2 at various doses. The amounts of IgM and IgG secreted by B cells were assessed by ELISA as described previously [19].

Proliferation assay

T cell cultures were stimulated with PHA, with or without E_2 and incubated for 3 days. [^{3}H]Thymidine ([^{3}H]Tdr, 0.2 μCi, 2 Ci/mmol, Amersham, UK) in 1 μl aliquots was added to each 20 μl well of the inverted Terasaki plates 2 h prior to harvesting and counting in a liquid scintillation counter [18]. Counts (dpm per well) from these microcultures are much lower than those of standard 200 μl cultures, but the ratio of response to background is superior.

Activation markers

Directly conjugated monoclonal antibodies used to assess lymphocyte surface antigens including activation markers by flow cytometry (Becton Dickinson FACSCAN) were: IL-2R α chain (CD25) FITC, Becton Dickinson (BD, UK); IL-2R β chain (p75) FITC, Endogen, USA; HLA-DR FITC, (BD) CD28 FITC, Immunotech, France; CD45RO FITC, Dakopatts, Denmark; CD45RA FITC, Dakopatts, Denmark. Cells were assessed by flow cytometry after 1 or 2 days of culture in the presence or absence of E_2 (0.2–100 ng/ml). Various cytokines and mitogens were used for the stimulation of the cell cultures (see Results). All the above markers were tested on $CD3^+$ ($CD4^+$ and $CD8^+$ subsets also), $CD19^+$ and $CD14^+$ cells. Antibodies for these cell subsets were direct conjugated with PE or PerCP (BD). The standard procedure of staining was carried out on ice. Data was assessed both by the percentage of cells positive for the particular antigen and by the level of expression per cell (mean channel).

Statistical analysis

Analysis of variance (ANOVA) of the data on immunoglobulin production and DNA synthesis was performed using a computerized statistical program (Genstat) to assess the treatment effects within tonsils. Other data were analysed by Student's t test.

RESULTS

Effects of E_2 on IgM secretion by human normal tonsillar B lymphocytes in the absence of other stimuli

The effect of E_2 was studied on B cell preparations with and without the addition of irradiated T cells, and the production of IgM and IgG compared. E_2 caused an increase in IgM production at all of its doses in the cultures when T cells were present (Fig. 1). In B cell cultures without added T cells the addition of most doses of E_2 had no effect. Only at a high dose of E_2 (20 ng/ml) was there a significant increase in IgM production in the absence of T cells.

Effects of E_2 on IL-2 driven IgM secretion by human normal tonsillar B lymphocytes

IL-2 was used to drive IgM secretion in a range of doses from 10–200 U/ml and E_2 was added at the same time as IL-2 at the start of the 8 day cultures. Table 1

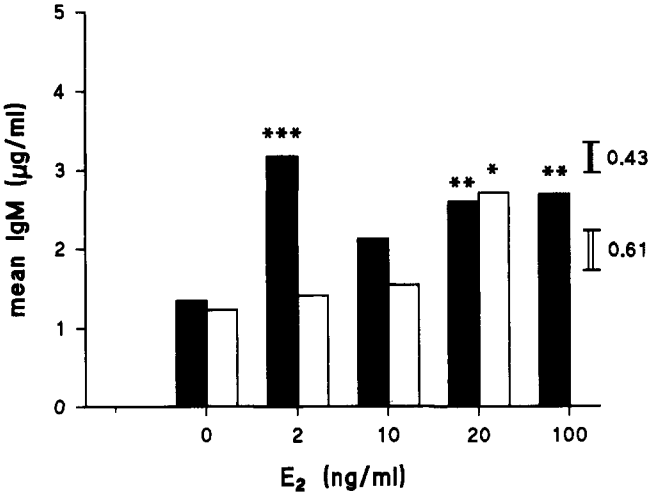

Fig. 1. Mean production of IgM (μg/ml) by high density tonsillar B cells in the presence (■) and absence (□) of irradiated T cells. (I) and (II) represent the standard error of the differences between the treatment means with or without T cells. These were calculated within tonsils using analysis of variance (Genstat). Significant differences between the treatment means with E_2 and the mean values in the absence of E_2 were tested by Student's t test ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$, at degrees of freedom of the error variance = 43 and 30 in the presence or absence of irradiated T cells, respectively). In the absence of T cells the dose of E_2 = 100 ng/ml was not tested.

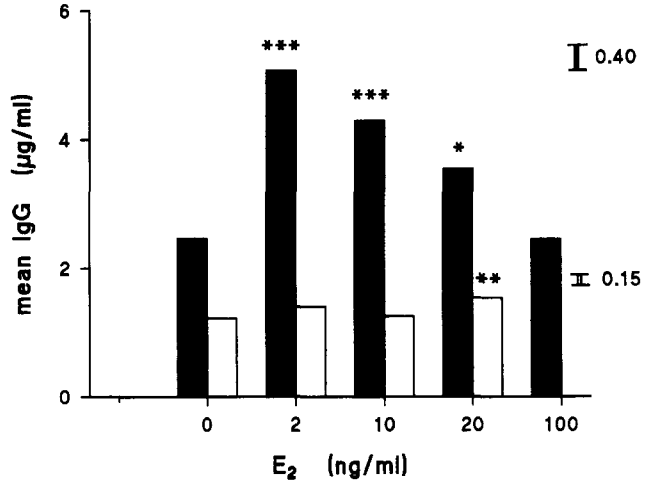

Fig. 2. Mean production of IgG (μg/ml) by high density tonsillar B cells in the presence (■) and absence (□) of irradiated T cells. Other details as in Fig. 1.

shows the effect of E_2 on IL-2 stimulated cultures of B cells without the addition of T cells [Table 1(A)] and with irradiated T cells present [Table 1(B)]. When T cells were not added, E_2 had little effect on IgM production of B cell cultures: however, a low dose of E_2 (2 ng/ml) caused a decrease in IgM production that was significant [Table 1(A)]. The significant effect of E_2 (at 10 ng/ml) in increasing IgM production induced by 10 U/ml of IL-2 was due to data from 1 out of the 4 tonsils. On the contrary, in the presence of T cells, E_2 significantly increased the IL-2 driven production of IgM [Table 1(B)].

Effects of E_2 on IgG secretion by human normal tonsillar B lymphocytes in the absence of other stimuli

Figure 2 illustrates the effect of E_2 on IgG production from B cell preparations with and without irradiated T cells in the absence of any added IL-2. The presence of irradiated T cells allowed E_2 to increase significantly the IgG production by these cultures.

Effects of E_2 on IL-2 driven IgG secretion by human normal tonsillar B lymphocytes

In the presence of IL-2, E_2 did not increase the IL-2 driven IgG production in the B cell cultures without T cells [Table 2(A)]. In some instances, E_2 caused a significant decrease in the IgG production from these cultures. However, when irradiated T cells were present and the cultures were stimulated with IL-2 at 100 or 200 U/ml, E_2 at some doses increased IgG production significantly [Table 2(B)].

Table 1. Effect of different doses of E_2 on the mean production of IgM (μg/ml) in tonsillar B cell cultures in the absence (A) or presence (B) of irradiated T cells in response to different concentrations of IL-2

IL-2 (U/ml)	E_2(ng/ml) 0	2	10	20	100	SED of means
			(A) Absence			
10	4.7	3.9	7.7*(a)	6.9	ND	1.1
50	11.8	7.2*(b)	9.1	8.9	ND	1.6
100	13.0	6.8***(b)	11.9	11.2	ND	1.6
200	14.0	6.5***(b)	11.2	10.5	ND	2.1
			(B) Presence			
10	4.9	7.7*(a)	6.5	6.1	7.6*(a)	1.1
50	10.9	10.1	17.0*(a)	12.7	16.0*(a)	2.6
100	10.9	10.9	19.9***(a)	13.8	17.4**(a)	2.2
200	11.4	12.3	19.4***(a)	14.5	18.5***(a)	1.9

(a) = increased and (b) = decreased at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ND = not done, SED of means = standard error of the differences between the means.

Table 2. Effect of different doses of E_2 on the mean production of IgG (μg/ml) in tonsillar B cell cultures in the absence (A) or presence (B) of irradiated T cells in response to different concentrations of IL-2

IL-2 (U/ml)	E_2(ng/ml) 0	2	10	20	100	SED of means
			(A) Absence			
10	2.3	2.6	2.6	2.7	ND	0.4
50	4.1	2.8**(b)	2.9**(b)	2.7**(b)	ND	0.4
100	4.7	3.3	3.1*(b)	3.5	ND	0.7
200	4.5	2.7*(b)	4.4	3.2	ND	0.9
			(B) Presence			
10	8.8	10.3	11.4	9.3	10.4	1.4
50	17.6	13.2*(b)	15.8	11.0**(b)	19.9	2.0
100	14.2	14.4	17.5*(a)	12.2	16.6	1.7
200	12.8	6.2*(a)	21.0***(a)	13.8	17.5**(a)	1.6

(a) = increased and (b) = decreased at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ND = not done, SED of means = standard error of the differences between the means.

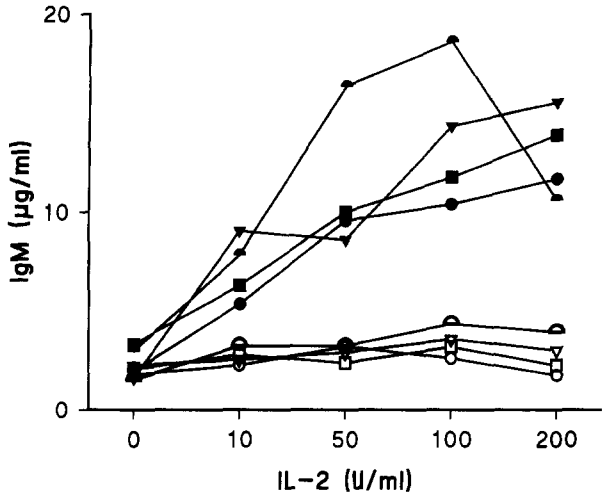

Fig. 3. Production of IgM (μg/ml) by high density tonsillar B cells when intact T cells were present (solid symbols) or when T cell supernatants were added (open symbols). The cells were stimulated with various doses of IL-2 (abscissa) and doses of E_2 (●, ○ 0 ng/ml; ■, □ 2 ng/ml; ▼, ▽ 20 ng/ml; ◓,◺ 100 ng/ml). The above graphs represent data from two different experiments with 6 replicates for each treatment condition.

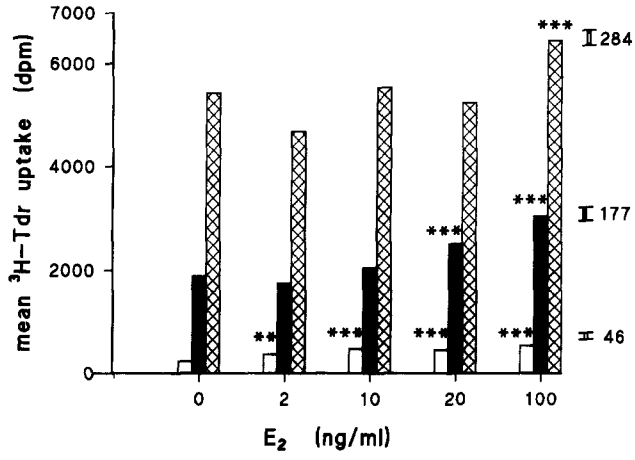

Fig. 5. Mean levels of the uptake of [³H]thymidine by tonsillar T cells stimulated with PHA (□, 0 μg/ml; ■, 0.5 μg/ml; ⊠, 1 μg/ml) and various doses of E_2 (abscissa). The degrees of freedom of the error variance were 100. The standard error of the differences between the treatment means were (II) 46, (I) 177 and (II) 284 dpm for the three doses of PHA; respectively. These were calculated within tonsils using analysis of variance (Genstat), (**$P < 0.01$; ***$P < 0.001$).

With 50 U/ml of IL-2 there was a significant decrease in the production of IgG after addition of E_2 at two doses.

Effect of E_2 on IgM production by B lymphocytes in the presence of irradiated T cells or T cell supernatants

Whether intact T cells are required for E_2 to act was tested by comparing the effects of adding T cells or T cell supernatants. IL-2 driven B cell cultures produced higher levels of IgM when intact irradiated T cells were present compared to when T cell supernatants were substituted for T cells (Fig. 3). In addition, the effect of E_2 was only seen when irradiated T cells were present but not when T cells were substituted with T cell supernatants. A similar pattern was followed for IgG production in the same cultures (Fig. 4).

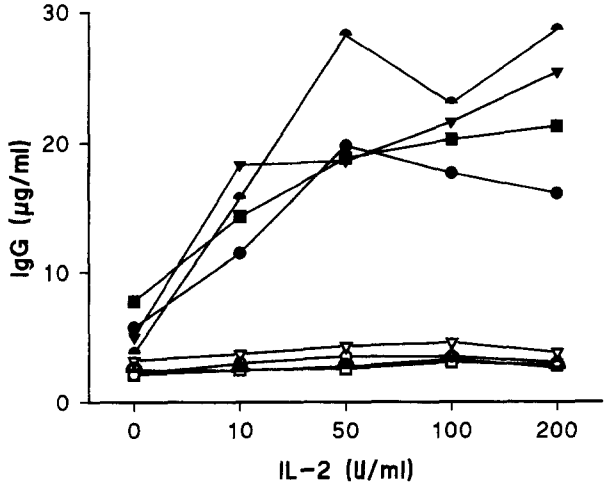

Fig. 4. Production of IgG (μg/ml) by high density tonsillar B cells when intact T cells were present (solid symbols) or when T cell supernatants were added (open symbols). Other details as in Fig. 3.

Role of E_2 on PHA-driven DNA synthesis by tonsillar T lymphocytes

To see whether the effects of E_2 on T-dependent IgM secretion could be reflected by an effect on T cell proliferation, doses of E_2 were added to T cell cultures. E_2 alone (at all doses tested) significantly increased the DNA synthesis by the T cells (Fig. 5). However, when the T cells were stimulated with PHA (0.5 or 1 μg/ml), the lower doses of E_2 had no effect on the DNA synthesis. Only the addition of high doses of E_2 (20 and 100 ng/ml) increased DNA synthesis significantly.

Effects of E_2 on surface antigens of tonsillar lymphocytes from normal donors

In order to explore the mechanism of the action of E_2 on lymphocyte function, the expression of a number of different lymphocyte surface antigens and activation markers were tested for changes after addition of E_2 to the lymphocyte cultures. These antigens were examined by flow cytometry on $CD3^+$ ($CD4^+$ and $CD8^+$ subgroups), $CD19^+$ and $CD14^+$ cells. In general, no effect of E_2 was discerned on these antigens either when E_2 was used alone or when the activation marker expression was increased by stimulation.

CD25 (IL-2 receptor, α-chain). This was assessed on $CD3^+$, $CD19^+$ and $CD14^+$ cells after they had been cultured for 2 days with different stimulants in the presence or absence of E_2 = 20 ng/ml. The stimulants used were: pokeweed mitogen (PWM, 1 μg/ml), IL-2 (100 U/ml), IL-4 (10 U/ml), IL-5 (10 U/ml), IL-6 (100 U/ml), γ-IFN (100 U/ml) and combinations including IL-2 and 6, IL-4 and 5, IL-6 and γ-IFN.

The presence of E_2 made no difference in the expression of CD25 on the above cell subsets. Moreover, T cell preparations cultured with OKT3 (1 μg/ml) showed no change in CD25 expression on $CD4^+$ and $CD8^+$ cells when 2 or 20 ng/ml of E_2 were added to the culture for 1 or 2 days. With PHA (0.5 or 1 μg/ml) there was a small increase in CD25 expression due to E_2 after 2 days of culture [Fig. 6(b)].

p75 (IL-2 receptor, β-chain). T cell preparations were cultured for 2 days with PHA (0.5 or 1 μg/ml) or with OKT3 (1 μg/ml) and the p75 antigen was assessed on $CD4^+$ and $CD8^+$ cells after the addition of $E_2 = 20$ ng/ml. There was no effect of E_2 on p75 expression. Additionally, B cells alone or B cells with irradiated T cells cultured with IL-2 (100 U/ml) and $E_2 = 20$ ng/ml, showed no change of p75.

HLA-DR (class II). B cells alone or cultures of B cells with T cells were stimulated with IL-2 (100 U/ml) for 2 days and tested for any changes in HLA-DR expression on the $CD4^+$, $CD8^+$ and $CD19^+$ subsets after addition of $E_2 = 20$ ng/ml. Again E_2 made no difference [Fig. 6(d)]. Also, tonsillar Ficoll-separated mononuclear cells cultured for 2 days with various doses of E_2 (0.2–100 ng/ml) alone, or with different cytokines including γ-IFN (100 or 1000 U/ml), IL-6 (100 U/ml) or combinations of γ-IFN and IL-6 (100 U/ml each), showed no changes of HLA-DR expression on $CD4^+$, $CD8^+$, $CD19^+$ and $CD14^+$ subsets after addition of E_2.

CD28. Tonsillar T cells were cultured with PHA (1 μg/ml) or PWM (1 μg/ml) for 1 or 2 days. Addition of E_2 (0.2–100 ng/ml) had no effect on CD28 expression on $CD4^+$ or $CD8^+$ subsets.

CD45RO/RA. The same cultures as for CD28 were tested for CD45RO and CD45RA expression after addition of E_2. No change was observed. E_2 had no effect on the T cell DNA synthesis or B cell

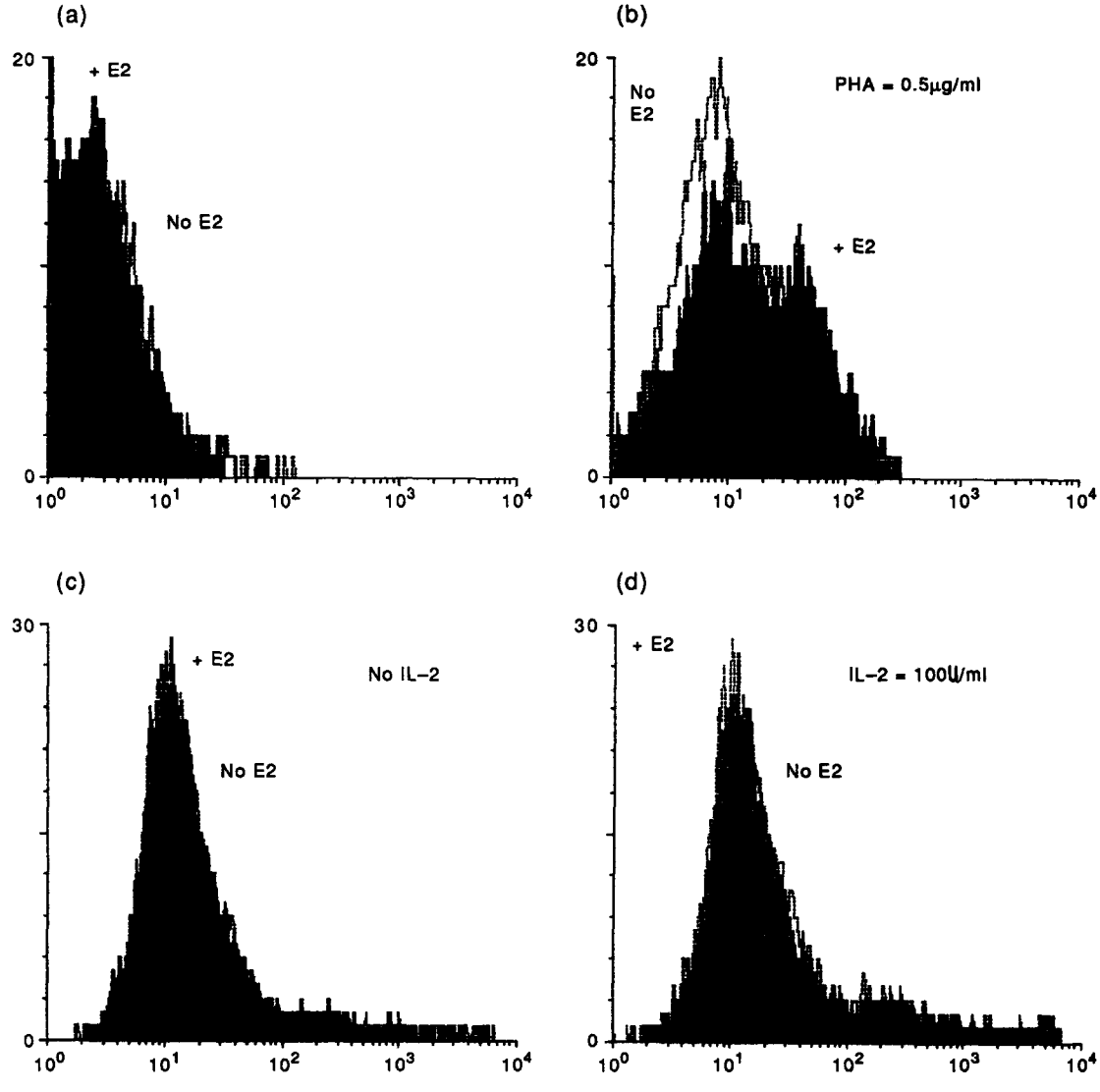

Fig. 6. Flow cytometry histograms showing the effect of E_2 (20 ng/ml) on the expression of IL-2R α chain (CD25) on $CD3^+$ cells without (a) and with (b) stimulation by PHA (0.5 μg/ml) and on the expression of HLA-DR on $CD4^+$ cells without (c) and with (d) stimulation by IL-2 (100 U/ml). On PHA stimulation there was a small increase in the expression of CD25 per cell in the presence of E_2 [see (b)].

immunoglobulin responses of any of the above stimuli used to modulate activation antigens other than the effects already reported.

DISCUSSION

To our knowledge, the present study is the first to examine the effects of E_2 on tonsillar lymphocytes. Our observations on tonsillar cells suggest that E_2 has an enhancing effect on lymphocyte differentiation and proliferation *in vitro*. E_2 at various doses significantly increased the background and IL-2 driven IgM and IgG production *in vitro* in a T-dependent way, observations which extend Paavonen's previous findings [2, 20]. He demonstrated that addition of E_2 to PWM-stimulated cultures of human PBL enhanced the IgM levels although there was no effect on B cell proliferation. However, when B cells were stimulated with *Staphylococcus aureus* there was no significant effect of E_2 which strongly suggests that the enhancing mechanism acts through T cells. Moreover, from our experiments the presence of intact T cells seems to be essential. It is not clear why E_2 induced a significant reduction in IgM secretion in our B cell cultures under some conditions. Perhaps this effect is due to a small number of residual T cells in the non-T preparation. Weetman's group [11] also examined the effect of E_2 on IgG synthesis by PWM-stimulated human PBL and found a significant increase in IgG plaque-forming cells in the presence of E_2 but only at physiological concentrations. Additionally, it has been reported that physiological concentrations of E_2 added to human PBMC significantly augment the Ag-specific immune response and enhance antibody production [21, 22].

Our present findings that E_2 alone at all doses tested exerts a significant enhancing effect on B lymphocyte IgM production seem to contradict partially with Kalman's data [10]. Although the cell preparations were different, his experiments with human PBL from normal male donors showed that E_2 alone had no effect but at physiological levels increased the numbers of IgM producing cells in PWM-stimulated cultures. Sthoeger [12] described a significant enhancement of PWM-induced generation of plaque formation cells by addition of physiological concentrations of E_2 *in vitro*. Bellini [13] also demonstrated a direct effect of E_2 on PHA-stimulated human PBL. However, pre-incubation of these cells with E_2 decreased significantly the cytosolic free Ca^{++} normally induced by PHA stimulation.

Accepting the different cellular preparations used, our observations on tonsillar cells agree with the above groups' work on human PBL, that E_2 has an enhancing effect on B cell differentiation through a T-cell dependent pathway. A possible mechanism for E_2 is through $CD8^+$ T cells (suppressor T cells) which express estrogen receptors in their cytoplasm [4, 5]. Inhibition of $CD8^+$ cells by E_2 may improve T helper cell activity which would enhance B cell maturation and subsequently increase immunoglobulin production [14].

We have clear evidence that E_2 added alone significantly increases DNA synthesis by tonsillar T cells at all doses tested. However, when the above cultures were stimulated also with PHA, only the higher concentrations of E_2 (20 and 100 ng/ml) induced a significant increase in DNA synthesis, data which coincide with Paavonen's [2] observations in human PBL. By contrast, various studies have reported that human PBMC stimulated with PHA or Con A showed no effect on DNA synthesis after addition of E_2 [15, 23, 24]. Other studies describe the inhibitory effect of supraphysiological doses of E_2 on PHA-stimulated lymphocyte proliferation [3, 11, 25, 26]. Our data cannot be compared directly with the above studies on human PBL since we used tonsillar T cells with distinctly different ratios of their subpopulations compared to blood.

Similar experiments with blood, splenic and thymic lymphocytes from mice and rats showed no response to E_2 [1, 14] or an enhancing dose related effect of E_2 [27].

The mechanism of action of E_2 was also studied by assessing whether it upregulates different activation markers and surface molecules on the tonsillar lymphocytes. Several possible mechanisms were unlikely since there was little effect of E_2 on any of these markers after 1 or 2 days culture, data which agrees with literature reports [15, 16].

Clearly, the mechanism by which E_2 modulates T and B lymphocyte function remains to be elucidated in more detail. It is clear that tonsillar lymphocytes are not as sensitive to low doses of E_2 as other systems e.g. breast cells. E_2 is known to affect the transcription of several genes in various cell populations after binding to its intracellular receptors. Now that we have established the action of E_2 in the tonsillar system, it will be useful to study the molecular mechanism of the action of E_2 on lymphocyte function in this system including its effects on cytokine gene transcription.

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