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Induction of Apoptosis in Thymocytes by Prostaglandin E_2 In Vivo

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In vivo administration in mice of a synthetic analog of prostaglandin E_2 (PGE_2) caused a selective and dramatic decrease of $CD4^+CD8^+$ double-positive, CD3/T-cell-receptor-αb^{lo} cells in the thymus. This loss was corticosteroid-independent and not affected by Cyclosporin A. The disappearance of $CD4^+CD8^+$ thymocytes was strictly correlated with the induction of apoptosis inside the thymus as shown by morphological studies and by the induction of intracellular transglutaminase expression. Considering that PGE_2 has been found to be produced by different cell populations inside the thymus, these results indicate that PGE_2 may act as endogenous signals for apoptosis during T-cell differentiation.

KEYWORDS: Prostaglandin E_2, apoptosis, thymocyte subsets, T-cell development, tissue transglutaminase.

INTRODUCTION

It is generally acknowledged that prostaglandin E_2 (PGE_2) exerts a powerful modulatory action on mature T cells (Goodwin, 1985; Vercammen and Ceuppens, 1987; Betz and Fox, 1991). Conversely, little information exists on its effects in the course of T-cell differentiation (Rinaldi-Garaci et al., 1983). The programmed cell death (PCD) of thymocytes is considered to be a crucial event that occurs during the intrathymic phase of T-cell differentiation. This death of physiological significance occurs in tissues by an active cellular phenomenon of self-destruction, called "apoptosis." It requires coordinate expression of regulatory proteins, as bc12, and enzymes, as Ca^{++}/Mg^{++}-dependent endonuclease and tissue transglutaminase (tTG), causing morphological modifications of the cell and leading to a final irreversible damage of DNA, characterized by molecule fragmentation (Wyllie et al., 1980; Arends and Wyllie, 1991; Piacentini et al., 1991a, 1991b). Apoptosis would lead to the controlled removal of thymocytes, during both positive and negative selection of functionally mature T cells (Rothemberg, 1990; Boyd and Hugo, 1991; von Boehmer, 1991; Zugic, 1991). The intrinsic mechanisms and the biochemical mediators of PCD in thymocytes in vivo are not yet clear (McConkey et al., 1990b). Moreover it is difficult to understand how T-cell-receptor (TCR)-mediated signals could result in two distinct thymocyte fates, that is, protection from PCD in the case of positive selection or induction of PCD in the case of negative selection. Recently, it has been demonstrated that an intracellular increase of cAMP stimulates PCD in rat and mouse thymocytes in vitro (McConkey et al., 1990a; Suzuki et al., 1991). Here we report the evidence that administration in mice of a synthetic analog of PGE_2, 16,16-dimethyl-PGE_2 methyl ester (DI-M-PGE_2), induces apoptosis of thymocytes in vivo, and that thymocytes at phenotypically different stages of differentiation show variable sensitivity to PGE_2.

RESULTS

Effects of PGE_2 Administration on Phenotypically Identified Thymocyte Subsets

DI-M-PGE_2, a long-acting synthetic analog of

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PGE_2, was administered i.p. into adult C57BL/6 mice at different doses in a single daily injection for a time ranging from 1 to 8 consecutive days. Thymuses were then collected and thymocytes were analyzed by flow cytometry using specific monoclonal antibodies. Results showed a dramatic and selective depletion of $CD4^+CD8^+$ cells. The effect was directly dependent on the dose, the number of the injections, and the time after the administration, with a maximum reached 12 hr after the last of four injections at the dose of 1 mg/kg/day (Figs. 1A to 1D). The prolongation of the treatment did not lead to further modifications. Regarding the expression of CD3 and TCR-$\alpha\beta$ molecules, thymocytes can be subdivided into negative cells and into low (lo), intermediate (int), and high (hi) expressing cells, corresponding to successive stages of T-cell differentiation (Ohashi et al., 1990). Dose-effect experiments demonstrated that CD3/TCR-$\alpha\beta^{lo}$ cells were the most sensitive to DI-M-PGE_2 action, followed by CD3/TCR-$\alpha\beta^{int}$, and negative cells (Figs. 1E to 1L). Conversely, CD3/TCR-$\alpha\beta^{hi}$, $CD4^+$, or $CD8^+$ single-positive thymocytes, expressing a "mature" phenotype, were highly resistant to DI-M-PGE_2. Looking at absolute numbers of thymocytes, a fall in all subsets in mice treated with DI-M-PGE_2 was observed, with the most dramatic change noted in the $CD4^+CD8^+$ cells. This was related to a decrease of thymus total cellularity after PGE_2 treatment (control diluent=$1.0\times10^8\pm0.1\times10^8$ cells, mean±SD, n=6; DI-M-PGE_2=$1.6\times10^7\pm2.0\times10^6$ cells, n=3, dosage= 1 mg/kg/day×4). The effect was reversible, as demonstrated by a good and progressive recovery of all subsets, which followed stopping treatment.

Effect of PGE_2 Administration on Tissue Transglutaminase Levels and on Morphological Features of Apoptosis in the Thymus

No evidence for a migration of $CD4^+CD8^+$ cells to peripheral lymphoid organs, after PGE_2 administration, as detected by flow cytometry at spleen and lymphonode level, was observed. We have then investigated if the disappearance of $CD4^+CD8^+$, CD3/TCR-$\alpha\beta^{lo}$, and thymocytes after DI-M-PGE_2 administration could be related to the intrathymic PCD of thymocytes that naturally occurs, particularly in cortical $CD4^+CD8^+$ thymocytes, during T-cell differentiation. Recently, in vitro and in vivo experiments have clearly demonstrated that apoptotic cells, both of normal and neoplastic origin, specifically express high levels of tTG (Fesus et al., 1987, 1989; Arends and Wyllie, 1991; Piacentini et al., 1991a, 1991b). By contrast, the enzyme expression is not enhanced during necrosis (Fesus et al., 1987). tTG, by catalizing covalent cross links between polypeptide chains, leads to the assembly of a stable protein scaffold (insoluble in detergents and chaotropic agents) that prevents the release of harmful molecules from the dying cell before its final degradation by phagocitosis (Fesus et al., 1989). The induction of tTG could so be considered as an early event during apoptosis. On the basis of these findings, we monitored the expression of tTG, in parallel with the morphology of thymus cells, in order to characterize apoptosis after DI-M-PGE_2 in vivo administration. Upon a single DI-M-PGE_2 injection, tTG activity was increased over the controls as early as 3 hr, reaching a two-fold increase at 24 hr. Repeated treatments had additive effects in enhancing the enzyme activity (Table 1). The effect of PGE_2 was limited to the thymus, as indicated by the absence of induction of tTG in other organs, such as spleen (control= 0.75±0.04 nmol/hr/mg protein, mean±SD, n=3; DI-M-PGE_2=0.35±0.06 nmol/hr/mg protein, n=3) or liver (control=0.62±0.05 nmol/hr/mg protein, n=3; DI-M-PGE_2=0.45±0.08 nmol/hr/mg protein, n=3). Immunohistochemical analysis of thymus

TABLE 1
Tissue Transglutaminase (tTG) Activity of Thymuses from DI-M-PGE_2 Treated Mice[a]

Treatment		tTG activity (% of control)
No. of injections (once a day)	Time after last injection (hr)	
1	3	150±12
1	12	183±41
1	24	198±35
1	48	161±30
1	72	155±24
2	3	298±52
3	3	315±48
4	3	277±36
2	24	212±37
3	24	225±45
4	24	214±30

[a]Transglutaminase activity was measured by detecting the incorporation of (^{3}H)putrescine into N,N'-dimethylcasein and calculated as nanomoles of (^{3}H)putrescine incorporated into protein per hour, expressed as a percentage from values obtained in mice treated with control diluent (0.24±0.08 nmol/hr/mg protein). Data are the cumulative means±SD of triplicate determinations of each individual thymus, derived from five different experiments (total n=30 for each experimental group).

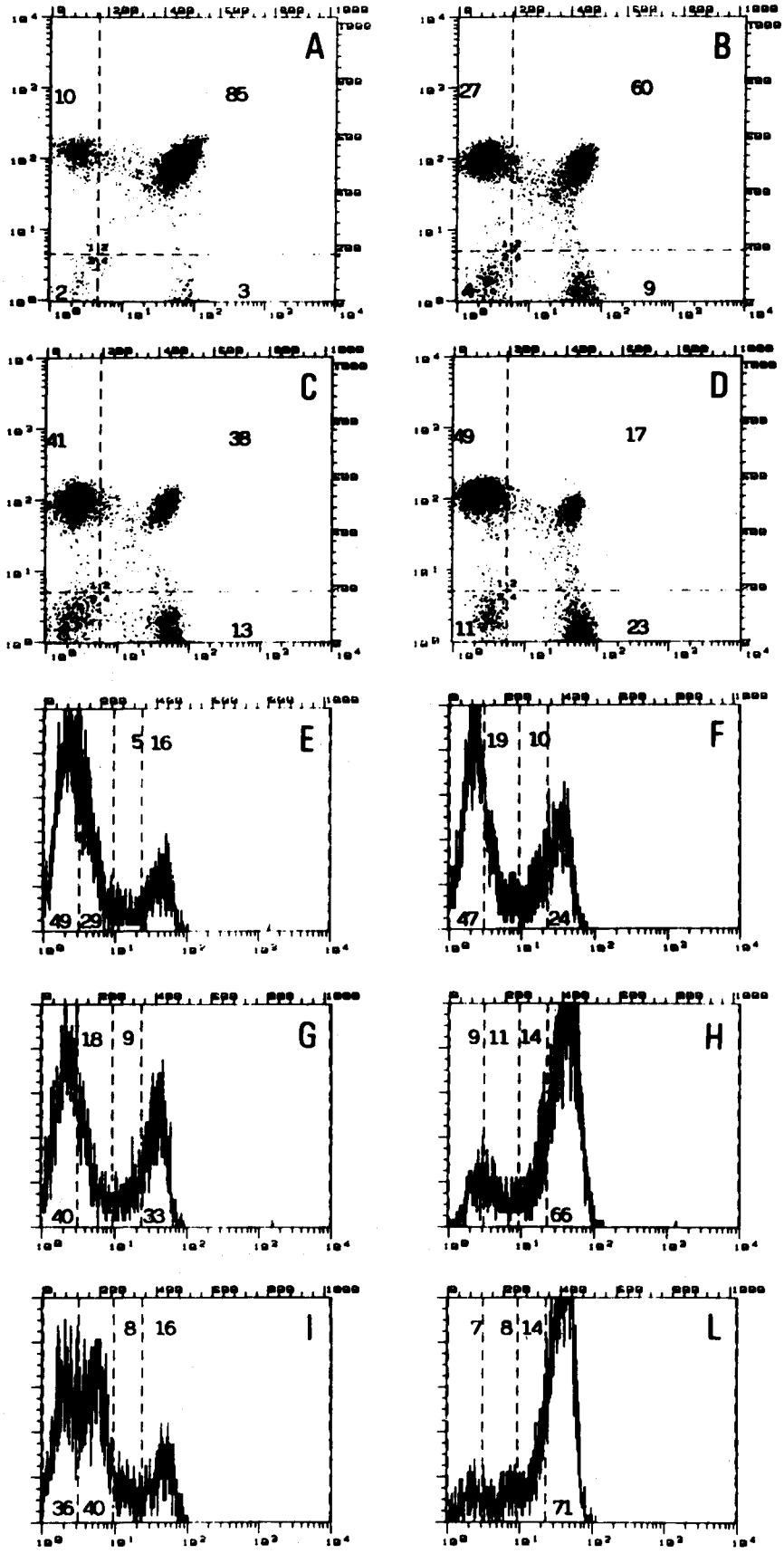

FIGURE 1. PGE_2 in vivo administration causes the selective loss of $CD4^+CD8^+$ and CD3/TCR-$\alpha\beta^{lo}$ thymocytes. C57BL/6NCr BR male mice were injected i.p. with control diluent (A, E, I) or 16,16-dimethyl prostaglandin E_2 (Di-M-PGE_2) at doses of 0.25 mg/kg (B and F), 0.5 mg/kg (C and G), and 1 mg/kg (D, H, and L) once a day for 4 consecutive days. Immunofluorescence staining and flow cytometry analysis were performed 12 hr after the last injection. The following antibodies were utilized: phycoerythrin conjugate antimouse L3T4 and fluorescein conjugate antimouse Lyt-2 for a two-color analysis of CD4 (vertical axis) and CD8 (horizontal axis) positive cells, respectively (A to D); fluorescein conjugate anti-CD3- for a single-color analysis (E to H); fluorescein conjugate anti-$\alpha\beta$ TCR (H57-597 mAb) for a single-color analysis (I and L). In single-color analysis, cell numbers (same full scale) are represented in the vertical axis and fluorescence, on the logarithmic scale, was plotted on the horizontal axis. The dashed lines in two-color analysis (A to D) indicate quadrant boundaries obtained by limiting 99.8% of the background events in the lower-left quadrant. The dashed lines in single-color analysis (E to L) indicate the upper and lower boundaries of CD3/TCR-$\alpha\beta$ lo, int, or hi populations for comparison among treatment groups. The first boundary was obtained by limiting 99.7% of the background events, and the others were set arbitrarily on the basis of the curve profile obtained in control samples. Numbers in the cytographs indicate percentages of cells within markers. The experiment was repeated six (A to D) and three (E to L) times, using three mice for each experimental group, with similar results within groups.

TABLE 2
Effect of PGE_2 Administration on CD4- and CD8-Identified Thymocytes in Adrenalectomized Mice[a]

Group	Treatment	Percent of total (mean±SD)			
		$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD4^-CD8^+$
1	None	1.6±0.6	83.5±1.0	10.0±1.0	4.7±0.6
2	Di-M-PGE_2	4.3±1.2[b]	55.6±13.0[b]	30.9±7.3[c]	9.2±4.5[d]
3	Adrenalectomy	1.2±0.6[d]	79.3±2.8[d]	11.6±1.8[d]	7.9±0.4[e]
4	Adrenalectomy plus Di-M-PGE_2	6.2±1.4[f,g]	54.9±7.3[f,h]	27.1±3.9[f,g]	11.8±2.0[f,i]

[a]Normal or adrenalectomized mice were injected with Di-M-PGE_2 at a dose of 0.25 mg/kg/day for 4 consecutive days (groups 2 and 4, respectively). Twenty-four hours after the last injection, thymuses were collected and flow cytometry analysis of thymocyte subsets was performed. Untreated (group 1) or adrenalectomized (group 3), sex, and age matched controls were also tested. Results represent percentage mean values±standard deviation obtained from three mice individually tested. Statistical analysis was performed by Student's *t*-test.
[b]$P<0.05$ against corresponding value of group 1.
[c]$P<0.01$ against corresponding value of group 1.
[d]N.S. against corresponding value of group 1.
[e]$P<0.005$ against corresponding value of group 1.
[f]N.S. against corresponding value of group 2.
[g]$P<0.005$ against corresponding value of group 3.
[h]$P<0.01$ against corresponding value of group 3.
[i]$P<0.05$ against corresponding value of group 3.

after DI-M-PGE_2 administration showed a large induction of tTG protein in several cells mainly localized in the thymus cortex (Figs. 2A to 2C). The morphology of positive cells showed the distinctive features of apoptosis (condensed chromatin and nuclear fragmentation) that appeared unequivocal when the immunostaining was performed in single-cell suspensions of thymocytes freshly isolated from DI-M-PGE_2 treated mice (Fig. 2D).

Effect of PGE_2 Administration on CD4- and CD8-Identified Thymocytes in Adrenalectomized Mice

Considering that a cell loss of $CD4^+CD8^+$ thymocytes, similar to that observed by us after DI-M-PGE_2 treatment, is also induced in vivo by glucocorticoids (Screpanti et al., 1989), we have investigated if the elimination of $CD4^+CD8^+$ thymocytes caused by PGE_2 could be mediated by endogenous steroid production. In fact, it is known that glucocorticoids induce apoptosis of thymocytes (Wyllie et al., 1980; Wyllie and Morris, 1982; Ojeda et al., 1990). Therefore, we have repeated the experiments in adrenalectomized mice. After 4 days of treatment at the dose of 0.25 mg/kg/day (the maximal tolerated dose of DI-M-PGE_2 by adrenalectomized mice), DI-M-PGE_2 administration resulted in a cell loss of $CD4^+CD8^+$ thymocytes, with respect to untreated controls, that was similar in intact as well as in adrenalectomized mice (Table 2). Thus, it is reasonable to hypothesize that PGE_2 action on thymocytes is not mediated by endogenous corticosteroids.

Effect of PGE_2 Administration on CD4- and CD8-Identified Thymocytes in Cyclosporin A-Treated Mice

It has been previously reported that apoptosis of thymocytes induced in vivo by anti-CD3 antibodies can be inhibited by Cyclosporin A (CsA) (Shi et al., 1989). In order to verify an eventual relationship between apoptosis induced in vivo

FIGURE 2 Immunolocalization of tTG protein in mouse thymus and freshly isolated thymocytes. Immunostaining both on paraffin included thymus sections and freshly isolated thymocytes was performed 3 hr after the last DI-M-PGE_2 injection using an affinity-purified monospecific rabbit IgG against soluble "tissue transglutaminase." Biotinylated goat antirabbit IgG was used as second antibody followed by a preformed avidin-horseradish peroxidase complex. Cells were counterstained in Mayer's hemalum. In thymus from control animals, the positivity to the anti-tTG antibody is limited to the endothelial cells lining the vessels (A). On the contrary, note the presence in the thymus cortex of many immunopositive (arrows) presumptive preapoptotic thymocyte after PGE_2 administration (B); Bar=60 μm. Higher magnification of the thymus cortex from PGE_2-treated animals (C); Bar=12 μm. Intense positivity to anti-tTG antibody is localized in cells showing a picknotic nucleus (arrows). Isolated thymocytes. After extensive washing in PBS, cells were smeared on slides, fixed in 2.5% paraformaldehyde, and, after immunostaining, counterstained with Mayer's hemalum. In thymocytes from control mice, no positive reaction was observed (data not shown); in cells isolated from PGE_2-treated animals, an increase in tTG protein expression is observed (D); Bar=60 μm. Note the intense staining of shrinked cells showing the condensed chromatin marginated at the cell periphery typical of apoptotic bodies (inset, Bar=12 μm). High concordant results were obtained in three independent experiments. (See Colour Plate VII at the back of this publication).

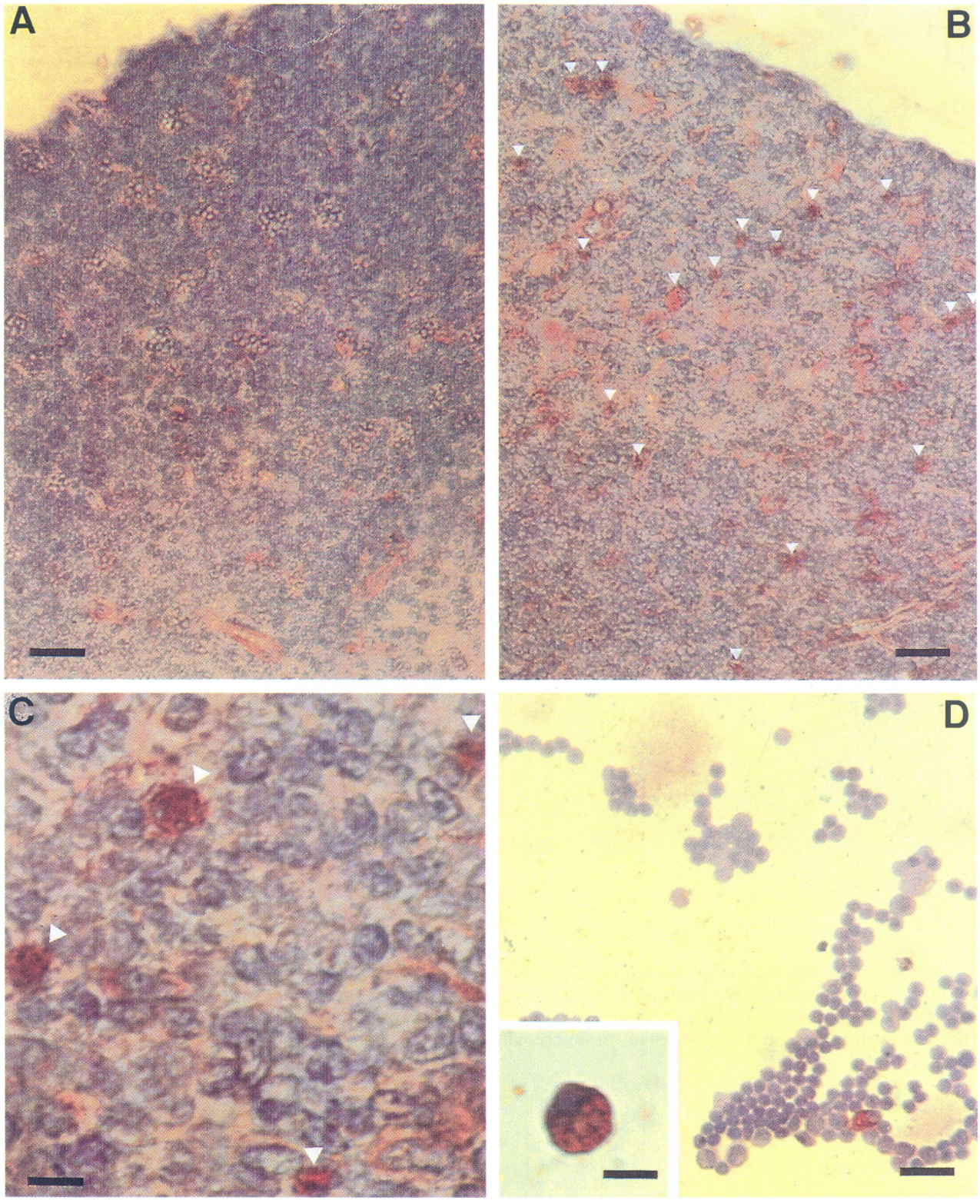

FIGURE 2.

by anti-CD3 antibodies and that induced by DI-M-PGE_2, we have investigated the effects of a simultaneous administration of PGE_2 and CsA. The flow cytometry analysis performed 24 hr after the last treatment did not show any difference between the cell loss of $CD4^+CD8^+$ thymocytes in mice treated with DI-M-PGE_2 alone and those treated with DI-M-PGE_2 plus CsA (Table 3). In addition, no changes in tTG activity were observed (data not shown).

DISCUSSION

A restricted number of experimental models of induction of thymocyte apoptosis has so far been described. In fact, apoptosis of thymocytes has been unequivocally proved to be induced in vitro by glucocorticoids (Wyllie et al., 1980; Wyllie and Morris, 1982; Ojeda et al., 1990), radiations (Yamada and Ohyama, 1988), calcium ionophores and phorbol ester (Kizaki et al., 1989), or in vitro as well as in vivo by anti-CD3 antibodies (Shi et al., 1989; Smith et al., 1989) and some peptide antigens (Jenkinson et al., 1989; Murphy et al., 1990). Our results provide the first direct evidence for a pharmacological induction of thymocyte apoptosis in vivo. In fact, it is described by a simple and highly reproducible pharmacological experimental model of thymocyte apoptosis in vivo. Moreover, the recently obtained evidence for the induction of thymocyte apoptosis in vitro by PGE_2 and other intracellular cAMP elevating agents (McConkey et al., 1990a; Suzuki et al., 1991) furnishes a clear in vitro experimental support to the observation we have obtained in vivo. The in vivo PGE_2-induced PCD includes pecularities that extend and clarify the previously described thymocyte apoptosis features. In fact, it is not mediated by endogenous glucocorticoids, nor inhibited by CsA as in vivo CD3-induced PCD. Indeed, glucocorticoids seem not to be produced inside the thymus, nor could radiations or anti-CD3 antibodies constitute physiological triggers for thymocyte apoptosis. On the contrary, PGE_2 are produced in large amounts inside the thymus by a wide variety of cell types as thymic epithelial cells, dendritic cells, macrophages (Gallily et al., 1985; Homo-Delarche, 1985; Nieburgs et al., 1985), and also nurse cells as recently reported (McCormack et al., 1991). Interestingly, a selective elimination of double-positive immature thymocytes by a thymic epithelial cell line has been reported in vitro (Nakashima et al., 1990); on the other hand, it has been recently demonstrated that thymic macrophages or dendritic cells are associated to different subsets of developing thymocytes, suggesting a role for thymic rosettes, consisting of thymocytes attached to a central stromal cell, in the maturation steps in the thymic cortex (Shortman and Vremec, 1991). Thus, it is possible that PGE_2, endogenously produced by stromal cells inside the thymus during the intercellular contacts, which seem to play an important role during T-cell development, could locally reach concentrations active to induce cell death of thymocytes by apoptosis. The different sensitivity of CD3/TCR-$\alpha\beta^{lo}$, CD3/TCR-$\alpha\beta^{int}$, or CD3/TCR-$\alpha\beta^{hi}$, thymocytes to PGE_2, could be involved in

TABLE 3
Effect of PGE_2 ADministration on CD4- and CD8-Identified Thymocytes in Cyclosporin-A-Treated Mice[a]

Group	Treatment	Percent of total (mean±SD)			
		$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD4^-CD8^+$
1	None	1.2±0.8	87.7±1.1	13.1±1.0	4.0±0.7
2	Di-M-PGE_2	3.9±1.2[b]	39.6±9.7[c]	37.1±9.7[d]	19.4±4.1[c]
3	Cyclosporin A	1.4±0.4[e]	82.8±2.2[e]	11.4±1.8[e]	4.4±0.6[e]
4	Cyclosporin A plus Di-M-PGE_2	6.1±1.6[f,g]	41.6±6.9[f,h]	32.2±2.8[f,h]	20.1±2.5[f,h]

[a]Mice were injected with: Di-M-PGE_2 at a dose of 0.5 mg/kg/day for 4 consecutive days (group 2); Cyclosporin A at a dose of 50 mg/kg/day for 4 consecutive days (group 3); both Di-M-PGE_2 plus CsA (group 4). Twenty-four hours after the last injection, thymuses were collected and flow cytometry analysis of thymocyte subsets was performed. A group of sex- and age-matched controls was also tested (group 1). Results represent percentage mean values±standard deviation obtained from three mice individually tested. Statistical analysis was performed by Student's *t*-test.
[b]$P<0.05$ against corresponding value of group 1.
[c]$P<0.005$ against corresponding value of group 1.
[d]$P<0.001$ against corresponding value of group 1.
[e]N.S. against corresponding value of group 1.
[f]N.S. against corresponding value of group 2.
[g]$P<0.01$ against corresponding value of group 3.
[h]$P<0.001$ against corresponding value of group 3.

the mechanism that leads to opposite final events (selection or removal by PCD) in different phases of T-cell differentiation. Our results could contribute to explain the mechanism involved in the physiological elimination of the majority of thymocytes at the $CD4^+CD8^+$ stage. A very rapid clearance by macrophages of apoptotic thymocytes could result in the difficulty to identify well the physiologically occurring phenomenon. On the other hand, the effects caused by PGE_2, at the pharmacological doses we have used, could overcome the capacity of phagocytic cells to eliminate dying cells, thus rendering the phenomenon as evident when using an early marker of apoptosis as tTG.

MATERIALS AND METHODS

Animals

Male C57BL/6NCrBR, 4-week-old mice, purchased from Charles River Italia (Como, Italy), and male C57BL/6J, 6-week-old adrenalectomized or sham-adrenalectomized mice, purchased from Nossan (Milan, Italy), were used.

Drugs and Treatment

Prostaglandin

Mice were injected i.p. with 16,16-dimethyl prostaglandin E_2 (Di-M-PGE_2) (Cayman Chem. Co., Ann Arbor, MI) at the doses of 0.25 mg/kg body weight, 0.5 mg/kg, and 1 mg/kg once a day for a time ranging from 1 to 4 consecutive days.

Cyclosporin A

CsA (Sandoz, Basel, Switzerland) was administered at the dose of 50 mg/kg of body weight, daily for 4 consecutive days, alone or immediately after Di-M-PGE_2 administration.

Immunofluorescence Staining and Flow Cytometry Analysis

Thymuses were individually processed 12 hr after the last injection by gentle teasing in RPMI 1640. The resultant cell suspension was filtered through a nytex mesh, washed twice with RPMI 1640, and resuspended in PBS at 2×10^7/ml cells. Immunofluorescence staining and flow cytometry analysis were then performed. The following antibodies were utilized: phycoerythrin conjugate antimouse L3T4 and fluorescein conjugate antimouse Lyt-2 (Becton Dickinson, Mountain View, CA) for a two-colors analysis of CD4- and CD8-positive cells, respectively; fluorescein conjugate anti-CD3-ε (clone 145-2C11) (Boehringer Mannheim Bioch., Mannheim, Germany) for a single-color analysis; fluorescein conjugate anti-$\alpha\beta$ TCR (H57-597 mAb), kindly provided by L. Jones (NCI, NIH, Bethesda), for a single-color analysis. Phycoerythrin conjugate antihuman CD4 and fluorescein conjugated antihuman CD8 (Becton Dickinson) were used as unrelated controls for background detecting. Staining was performed at 4 °C for 30 min. After treatment, cells were washed twice in PBS containing 0.02% sodium azide and flow cytometry analysis was performed using a FACScan (Becton Dickinson). In two-color analysis, marks were set to indicate quadrant boundaries limiting 99.8% of the background events in the lower left quadrant. In single-color analysis, markers were set to indicate the upper and lower boundaries of CD3/TCR-$\alpha\beta$ lo, int, or hi populations for comparison among treatment groups. The first boundary was obtained by limiting 99.7% of the background events, and the others were set arbitrarily on the basis of the curve profile obtained in control samples and maintained in the experimental samples. Data collection was gated on live thymocytes by forward and side angle scatter, utilized to exclude dead cells, debris, very large nonlymphoid cells, and cell aggregates. Data represent 5000 events.

Immunocytohistochemistry

Immunostaining on paraffin included thymus sections and freshly isolated thymocytes was performed 3 hr after the last DI-M-PGE_2 injection using as primary antibody (diluted 1:100) an affinity-purified monospecific rabbit IgG raised against soluble "tissue transglutaminase" of human red blood cells (kindly furnished by L. Fesus, University Medical School of Decebren, Hungary) in a wet chamber overnight at 4 °C. Biotinylated goat antirabbit IgG was used as a second antibody followed by a preformed avidin-horseradish peroxidase complex (Immunon, Detroit, MI). The reaction was devel-

oped using aminoethylcarbazole as chromogen substrate and 0.01% H_2O_2. Cells were counterstained in Mayer's hemalum. Endogenous peroxidase activity was blocked by methanol-H_2O_2. Isolated thymocytes were obtained as previously described for flow cytometry analysis. After extensive washing in PBS, cells were smeared on slides, then fixed in 2.5% paraformaldehyde and, after immunostaining, counterstained with Mayer's hemalum.

Tissue Transglutaminase Activity

Thymuses were collected from control or Di-M-PGE_2, at various time intervals after PGE_2 administration, extensively washed in PBS, and homogenized in 0.1-M Tris-HCl, pH 7.5, containing 0.25-M sucrose, 0.5-mM EDTA, and 1-mM PMSF. Transglutaminase activity was measured by detecting the incorporation of (^{3}H)putrescine into N,N'-dimethylcasein. The incubation mixture contained 150-mM Tris-HCl buffer, pH 8.3, 5-mM $CaCl_2$, 10-mM dithiothreitol, 30-mM NaCl, 2.5-mg N,N'-dimethylcasein/ml, 0.2-mM putrescine, containing 1-mCi (^{3}H)putrescine, and 0.1–0.2-mg protein in a final volume of 0.3 ml. After 20 min of incubation, the mixture was spotted onto Whatman 3 mm filter paper moistened with 20% trichloroacetic acid (TCA). Free (^{3}H)putrescine was eliminated by washing with large volumes of cold 5% TCA containing 0.2-M KCl before counting. tTG activity was calculated as nanomoles of (^{3}H)putrescince incorporated into protein per hour, and was expressed as a percentage from values obtained in mice treated with control diluent.

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