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# DEXAMETHASONE AND 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN CAN INDUCE THYMIC ATROPHY BY DIFFERENT MECHANISMS IN MICE

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SUMMARY The effects of in vivo exposure to dexamethasone (DEX) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on thymocyte proliferation and thymocyte number were compared. In the thymus of DEX-treated mice (1 mg/kg) both proliferation and cell number had decreased by 70 % one day after exposure. This decrease was, however, transient, and values returned to normal within 2 weeks. By contrast, in TCDD exposed mice (50 μg/kg), a reduction in proliferation was not observed until day 2 after exposure, and the degree of reduction was only about 50 %. By this point in time, cell number had only decreased by 20 %. Proliferation increased again on day 3 after TCDD administration, whereas cell number continued to decrease and remained low throughout the observation period (8 days). DEX had a direct and immediate effect on cells in all thymocyte subpopulations whereas TCDD initially only affected the immature double negative (DN) and double positive (DP) populations. 

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), an environmental pollutant and the most toxic compound among a large group of halogenated aromatic hydrocarbons, can have profound immunotoxic effects on experimental animals. In many respects these effects resemble the immunosuppressive effects caused by corticosteroid hormones. Both TCDD and corticosteroids can reduce resistance to a number of bacterial and viral infections, suppress antibody (ab) production, affect cell-mediated immunity, and reduce lymphoid tissues, particularly the thymus (1, 2). In addition, the effects of TCDD and steroid hormones are mediated through their binding to specific intracellular receptors with structural and functional similarities (3-5).

Concerning thymic atrophy, it has been suggested that both glucocorticoids and TCDD kill immature thymocytes by initiating programmed cell death (PCD), and that this cell-killing effect is independent of any interference with the thymic stroma (6, 7). By contrast, other groups have claimed that rather than TCDD-induced thymic atrophy being caused by direct effects on the thymocytes, it is mediated via a disturbance of the thymic epithelial- and dendritic cells (8-10). In addition, we showed earlier that even with a high dose of TCDD (50 μg/kg) the main loss of thymocytes in vivo does not occur before 2 - 4 days have elapsed since TCDD exposure (11), suggesting that a direct effect on thymocytes is unlikely to occur in vivo. In the present study, TCDD and the glucocorticoid dexamethasone (DEX) were compared in terms of their effects on cell proliferation, cell number in different thymocyte subpopulations, and on MHC class II expression on thymic epithelial cells.

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## MATERIALS AND METHODS

Chemicals and animal exposure. TCDD (98% pure, Cambridge Isotope Laboratories, Woburn, Mass., USA) was dissolved in 1,4-dioxane and subsequently diluted with corn oil (1:4 v/v). DEX (a gift from Merck Sharp & Dohme AB, Bromma, Sweden) was dissolved in distilled water at a conc. of 1 mg/ml. Two to four-week-old male C57BL/6 mice, weight and age matched and randomly allocated to treatment groups, were injected intraperitoneally (i.p.) with either DEX at a dose of 1 mg/kg body weight (b.w.) or TCDD at a dose of 50 μg/kg b.w.. The total volumes injected were approximately 5 μl per mouse. Control mice were administered the vehicles only.

Assessment of cell proliferation was done in 3 to 4-week-old mice at 1, 2, 3, 4, 6, 8, and 14 days after exposure to DEX or TCDD. Two hours before sacrifice, the animals were injected with $^{3}$H-thymidine, 200 MBq/kg b.w. (Amersham International plc, Amersham, England; spec. act., 0.74-1.1 TBq/mmol). Single cell suspensions were made, and after counting the cells, radioactivity was measured as described elsewhere (12).

Flow cytofluorometic analysis and immunoperoxidase staining. Different thymocyte subpopulations can be identified based on their expression of L3T4 (CD4) and Lyt-2 (CD8) molecules (13). The effects of DEX and TCDD on these populations and on the amount of expressed antigen were studied at 1, 2, 3, and 4 days after exposure of 2-week-old mice by using monoclonal antibodies (Mab) against these molecules and a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif., USA) as described previously (11). Phycoerythrin (PE)-conjugated rat anti-mouse L3T4 (CD4) ab, Fluorescein (FITC)-conjugated rat anti-mouse Lyt-2 (CD8) ab, and PE-conjugated streptavidin were bought from Becton Dickinson. Four days after TCDD exposure, thymocytes were also stained with biotinylated hamster anti-mouse αβ T cell receptor (TCR) ab (14). The MHC-class II expression on thymic epithelial cells was investigated at various times after exposure (10 h-10 days) to the two compounds. The thymuses were removed and frozen in hexane plus dry ice and stored at -70° C. After freeze-sectioning the tissue was air dried and fixed in acetone. Immunoperoxidase staining was performed using a mouse anti-MHC class II ab (I-$A^{b}$), clone 7-16.17, a kind gift from Dr. R. Holmdahl, Uppsala University, and the Avidin-Biotin method.

Uptake of $^{14}$C-TCDD in the thymus. $^{14}$C-TCDD (Cambridge Isotope Laboratories, Woburn, Mass. USA ; spec. act., 4.7 TBq/mmol TCDD) was injected i.p. into mice at a dose of 50 μg/kg b.w.. On each of four days (1, 2, 4, and 8 days after exposure) three mice were sacrificed and their thymuses removed and weighed. Soluene 350® and Toluene Scintillator® were added before measuring radioactivity in a liquid scintillation spectrometer.

## RESULTS

*Cell proliferation and thymocyte number after DEX- and TCDD-treatment.* Both the DEX- and TCDD-treatments transiently inhibited thymocyte proliferation; however, they differed in terms of the magnitude and duration of the inhibition induced. In DEX-treated mice the decrease in proliferation paralleled the reduction in cell number, and both parameters reached a minimum within 24 h (about 30% of that in controls) (Fig. 1). In addition, a hypocellular thymus cortex became evident in freeze-sections within 10 h after DEX-treatment. The rate of proliferation increased again 2 days after exposure which led to an increase in cell number by day 4. Both proliferation and cell number had returned to control levels after 14 days.

In TCDD-exposed mice, the inhibition of cell proliferation preceded the decrease in cell number. Maximal inhibition was not observed until 48 h after exposure and by this point in time cell number had only been reduced by 20%. In addition, the degree of inhibition was less pronounced than that observed after DEX-exposure (about 50% compared with controls). The subsequent increase in cell proliferation occurred more slowly than in DEX-treated mice and did not lead to an increase in cell number. The minimum number of thymocytes, 15% of that in control mice, was reached 4 to 6 days after treatment.

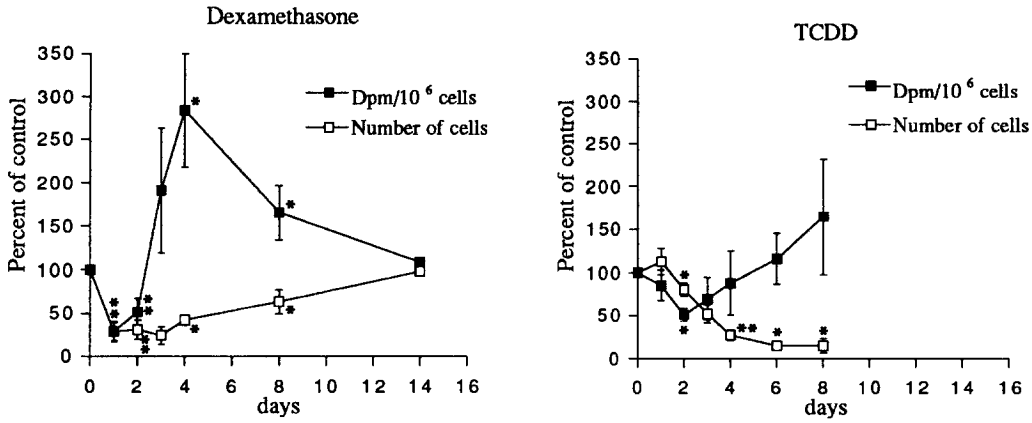


Figure 1. Proliferation and cell number in the thymus after DEX- and TCDD-exposure. C57Bl/6 mice, 3-4 weeks of age, were injected i.p. with single doses of either DEX (1 mg/kg), TCDD (50 μg/kg) or the corresponding vehicles only (controls). At 1, 2, 3, 4, 6, 8, and 14 days after treatment, the mice were injected with $^{3}$H-thymidine (200 MBq/kg body weight) and sacrificed 2 h later. The thymuses were removed, the cells counted, and the radioactivity was measured. Values are means from at least three independent experiments with three mice in each group ± S.D. In DEX-treated mice, the number of cells was reduced by 70% on day 1 ($p < 0.05$) (hidden in the figure).

p values were calculated with the Student's t-test.

* $p < 0.05$; ** $p < 0.01$

*Effects on thymocyte subpopulations and MHC-class II expression in thymuses from TCDD- and DEX-treated mice.* DEX-treatment resulted in a decrease in cell number in all thymocyte populations already one day after exposure (Fig. 2). Reductions were most pronounced in the immature double negative (DN) $CD4^{-}CD8^{-}$ and double positive (DP) $CD4^{+}CD8^{+}$ populations, decreasing from ca 5 million to 2 million and from 140 million to 40 million cells respectively. Thereafter the DP and $CD4^{+}$ populations continued to decrease until day 3 after exposure, whereas DN and $CD8^{+}$ populations increased over the next 2 to 3 days. The number of $CD4^{+}$ cells did not increase during the 4-day period, nor was such an increase expected since the turnover rate of DP cells is about 3-4 days (15).

TCDD-exposure led to a decrease in cell number in mainly the DN and DP populations; however, it occurred at a later stage compared with the DEX-treated mice. In these DN and DP populations a small, but statistically significant, decrease in cell number had become evident by day 2 after exposure. By day 4, numbers of DN and DP thymocytes had reached levels as low as those obtained in the DEX treated mice on days 1 and 2. The number of $CD4^{+}$ thymocytes did not reach a level significantly lower than that in the controls until day 3 after TCDD exposure. The number of $CD8^{+}$ cells in TCDD-treated mice was significantly lower than controls on day 2 but not on day 3 and 4. No increase in cell number was observed during the experimental period in the DN, DP, and $CD4^{+}$ populations.

The amounts of CD4 and CD8 molecules, expressed per cell, after DEX- or TCDD-treatment were the same as those in the corresponding control at all observation points, and the expression of the αβ TCR 4 days after TCDD-exposure was also similar to that in the controls (data not shown).

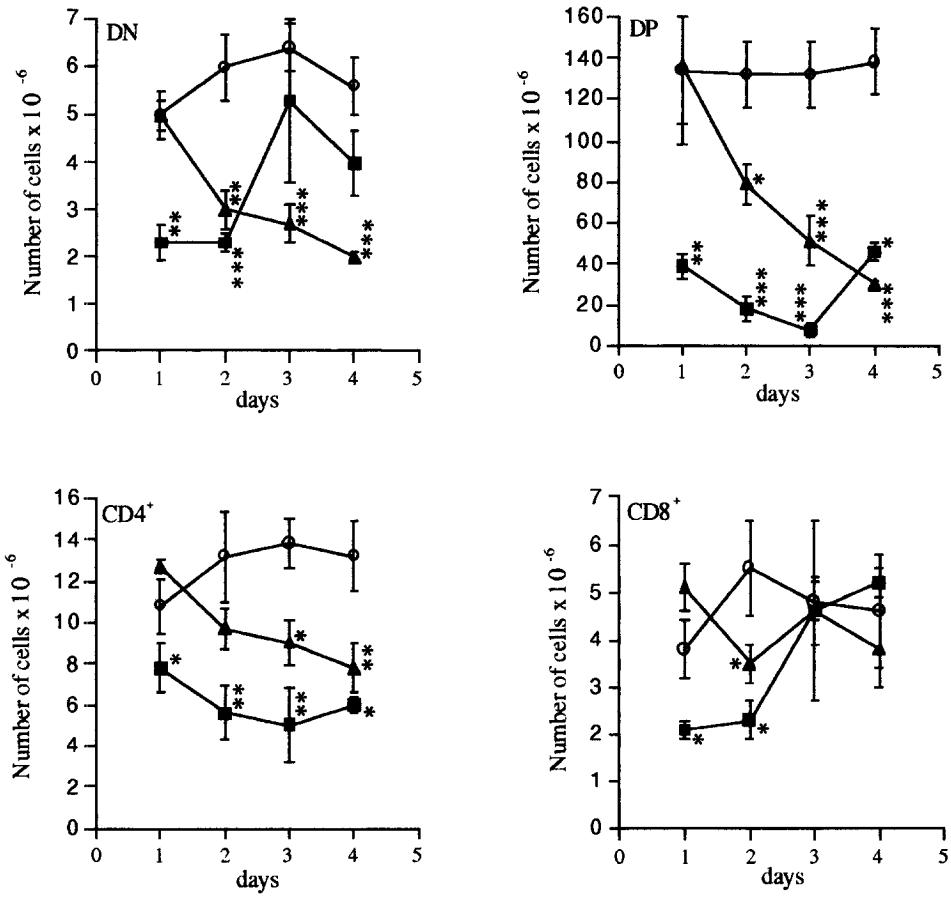


Figure 2. Number of cells in different thymocyte subpopulations after DEX- and TCDD-treatment. Mice were injected i.p. with single doses of DEX (1 mg/kg)(■), TCDD (50 μg/kg)(▲), or with the corresponding vehicles only (controls)(o). At 1, 2, 3, and 4 days after exposure the mice were sacrificed and the thymuses were removed. Thymocytes were counted and labelled with monoclonal antibodies against the CD4 and CD8 antigens and then analysed in a FACScan. Each point represents a mean of three independent experiments with three mice in each group ± S.D.
p values were calculated with the Student's t-test
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Within 10 h after the DEX-treatment there had been a slight increase of the MHC class II expression in the cortex (Fig. 3). By day 3 it had increased further, but 10 days after exposure there were no differences compared with controls. TCDD-exposure also increased the MHC expression in the cortex, however not before 3 days after treatment. On day 10, the MHC class II expression was very high, particularly in the subcapsular part of the thymus.

*Uptake of $^{14}C$-TCDD in the thymus.* TCDD reached the thymus quickly after adminisration to the mice. Administering a dose similar to that used in the previous experiments (50 μg TCDD/kg b.w.) resulted in a TCDD concentration of 4.8 ng/g thymus weight one day after exposure (first observation point). This concentration remained about the same throughout the 8-day study period (range 3.4-5.8 ng/g thymus weight).

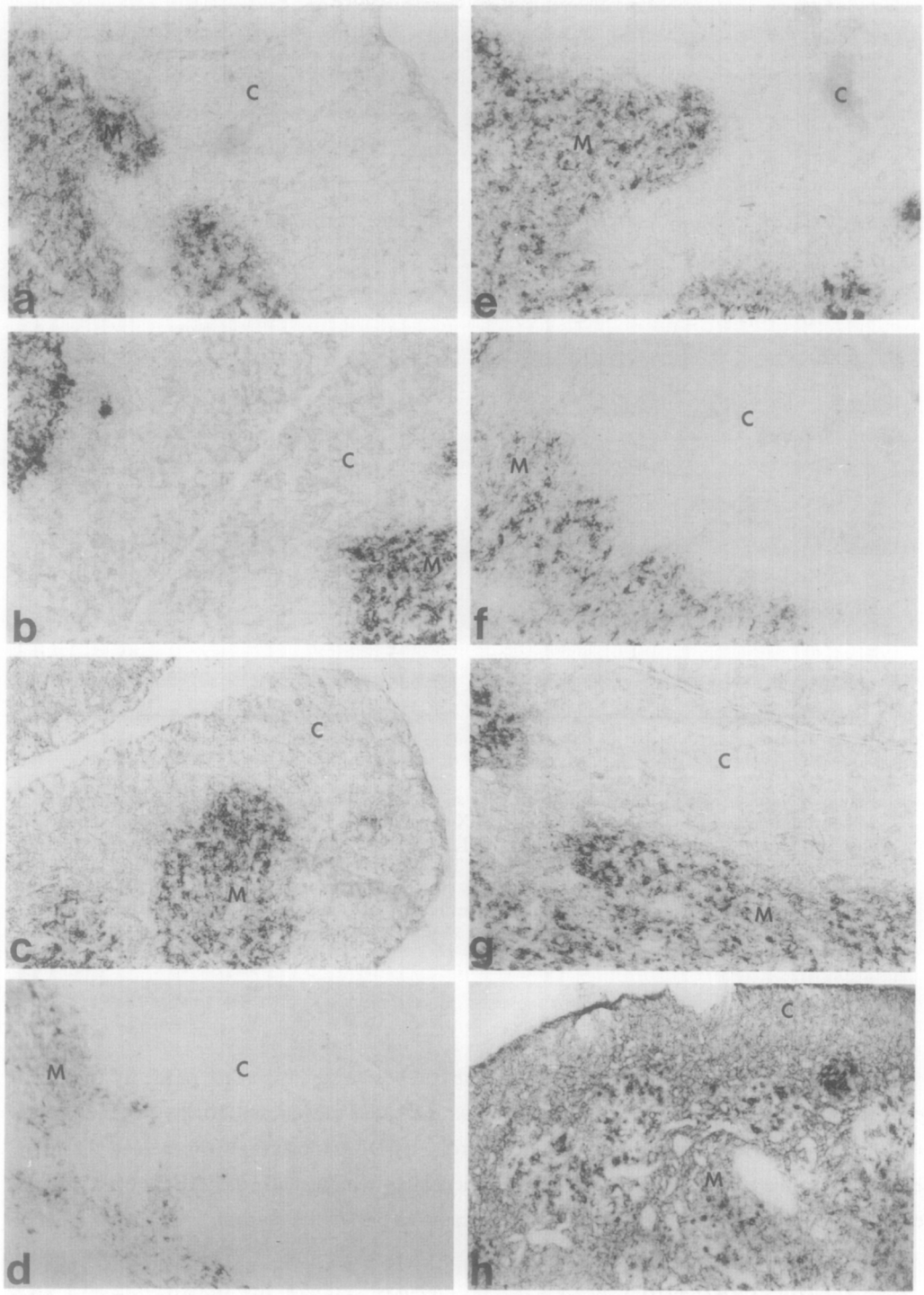


Figure 3. Immunoperoxidase staining of thymic freeze-sections of C57Bl/6 mice with mouse anti - MHC class II ab. Mice were either treated with DEX (1mg/kg) and their thymuses removed after (b) 10 h , (c) 3 days and (d) 10 days, or with TCDD (50 µg/kg) whereafter thymuses were removed at (f) 24 h, (g) 3 days and (h) 10 days. Controls for DEX (a) and TCDD (e) were treated with the corresponding vehicle only. No differences were observed between the two control groups at any point in time. Abbreviations: C = cortex; M = medulla. (x 50)

## DISCUSSION

It has earlier been demonstrated that there are many similarities between glucocorticoids and TCDD, and it has been suggested that the two compounds induce thymus atrophy in similar ways (1-5). In this study, however, differences were found between the DEX- and TCDD treatments in the time at which cell depletion first became detectable and in the time required before cell number began increasing again. In addition, the kinetics of the initial decrease and subsequent increase in cell proliferation was also different. It also seems clear from this study and others (16) that TCDD enters the thymus quickly, i.e. within hours after parenteral administration. Thus differences in time elapsed between administration and cell depletion cannot be explained by differences in the uptake and distribution of the two compounds. However, the fact that TCDD remains in the thymus for a long period of time, whereas DEX has a very short half-life, counted in hours rather than days (17), may account for the long-term differences observed in this study.

In a normal thymus only a few percent of the DP thymocytes will survive to maturity. It is believed that these cells are not only rescued from PCD by positive selection but are also able to avoid deletion through negative selection, which also involves PCD (18-21). Thus, as proposed by others (6, 7), it seems reasonable that DEX- or TCDD-exposure, at least partly, induce PCD in DP thymocytes, since this appears to be the normal route of cell death in the thymus. A crucial question to be answered, however, is whether the two compounds induce PCD by directly affecting the thymocytes and/or via interactions with the thymic stroma.

In young mice, only 10-20 % of the thymocytes are proliferating at any given time (15), and the phenotype of these proliferating cells is either DN or DP. Thus the sharp decrease in DN and DP cells after DEX-exposure may explain why proliferation was simultaneously low. Soon after the proliferation rate had started to rise, the cell number began to increase, reaching control values within 2 weeks. A similar cell-number regeneration kinetic was observed after DEX-treatment (22), antimitotic drug treatment (23), and sublethal irradiation (24, 25), treatments which are thought to kill most thymocytes without damaging the thymic stroma or intrathymic precursor cells. Thus the relatively rapid changes in cell number after DEX-treatment suggest that DEX can have a direct cytotoxic effect on the thymocytes without necessarily interfering with the thymic stroma. This is also supported by the rapid decrease in the mature $CD4^+$ and $CD8^+$ populations, which normally have a turnover rate of at least 3-4-days (15, 23) and which are not likely to enter PCD. The early and rapid increase in cell number in the $CD8^+$ population was probably caused by the development of immature $CD8^+$ thymocytes which represent an intermediate stage between DN and DP cells (23, 26). It has earlier been shown that DEX can alter the morphology of the cortical stroma and can have minor effects on MHC-expression (27), which may, however, be a consequence of thymocyte depletion.

In TCDD-treated mice proliferation decreased before a reduction in cell number was observed, indicating that the reduced proliferation was not caused by cell death. Furthermore, although thymocyte proliferation increased again 2 days after TCDD exposure, numbers of DN and DP cells continued to decrease and stayed low for at least 20 days after TCDD exposure (11). Selection can occur at different stages of DP-cell differentiation (28, 29). A TCDD-induced shift of the selection process to an earlier stage in the differentiation pathway could explain why cell

number remained low even after cell proliferation had returned to normal levels. Thus, TCDD may induce thymic atrophy both by inhibiting cell proliferation and by disturbing the normal selection processes, possibly through interference with the thymic stroma, which has been reported elsewhere (8-10). The marginal effects of TCDD on the number of mature $CD4^+$ and $CD8^+$ thymocytes support the hypothesis that TCDD interferes with the thymic stroma rather than having direct effects on thymocytes. The minor decrease in the $CD4^+$ population that became evident 3-4 days after treatment, could have been the result of the exhaustion of these cells due to their emigration to the periphery, in parallel with a decreased input of new cells from the DP pool.

Effects on bone marrow prethymocytes have been suggested to be involved in the development of TCDD-induced thymic atrophy (30). However, the influx of prethymic cells into the thymus seems to be low (15), and since DN, proliferating precursor cells remain within the thymus, an effect of TCDD on prothymocytes would probably only influence thymocyte number in the long-term perspective.

A prerequisite for normal maturation in the thymus is that interactions occur between the TCR/CD3-complex, the CD4 and the CD8 molecules expressed on thymocytes, and the MHC determinants on the thymic stroma (28, 31-33). Neither compound had any observed effects on the amount of CD4 and CD8 per cell, or in the case of TCDD on the amount of $\alpha\beta$ TCR per cell. By contrast, the MHC class II-expression increased, but not before the first signs of cell depletion had become evident. Thus, rather than increased MHC-expression being the reason for the induced cell depletion, it may be viewed as an attempt to increase the positive selection and thereby regain balance in the thymus.

The results of this study indicate that although there are many similarities between DEX and TCDD in terms of the symptoms they cause in vivo (P-450 induction, thymic atrophy etc.) and their effects at a biochemical level (similar intracellular receptors), their mechanisms of induction of thymic atrophy may nonetheless be different.

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