Induction of IL-2 by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in fetal thymocytes and mature T-cells of the mouse

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Abstract
We have detected three consensus „dioxin-responsive elements" in the promoter region of the interleukin 2 (IL-2) gene. These sequences are capable of specifically binding to the Ah-receptor as shown by band shift assays. IL-2 is upregulated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the developing thymus in fetal thymus organ culture. The increase is due to the CD4-CD8- and the CD4-CD8+, CD4+CD8- cells, but not the CD4+CD8+ or stromal cells. Moreover, IL-2 RNA is increased in the thymi of mice six days after injection of 50µg/kg TCDD body weight. No increase in total spleen was found under these conditions. When isolated thymocytes or spleen cells were stimulated with anti-CD3 antibodies, which provides signals for T-cell activation, TCDD synergistically increased the IL-2 production. Thus, TCDD may interfere with the signalling cascade leading to IL-2 production, either by direct transcriptional activation of the IL-2 gene, or by short-circuiting the second signal necessary for T-cell activation, possibly by modulation of c-jun.

Introduction
Among the most prominent effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in experimental animals are thymus atrophy and systemic immunosuppression. The latter is found for both the humoral and cellular immune response, with T-cell function affected at extremely low doses. Interleukin-2, which is produced by T-cells, is a key interleukin and has a central function for growth and differentiation of T-cells, B-cells, natural killer cells, macrophages and others. As indicated from studies in gene-targeted mice, complete lack of IL-2 leads to uncontrolled activation and proliferation, autoimmune disease, and premature death. Apparently, IL-2 is involved in T-cell homeostasis and can also negatively regulate T-cell growth. We had previously identified by computer analysis three putative consensus sequences of „dioxin responsive elements" in the 5'region of the IL-2 gene[1]. Also, we had dectected a threefold upregulation of IL-2 RNA during fetal thymus development upon exposure to TCDD in fetal thymus organ cultures[2]. We test here whether the consensus DRE-sequences in the IL-2 promoter can bind to the Ah-receptor. Moreover, since fetal thymocytes conceivably are different from adult thymocytes or T-cells with respect to their signalling requirements for interleukin production, we analyse whether conditions for a TCDD-induced modulation of IL-2 might be found for the adult thymus and peripheral T-cells.

Experimental methods
Mice of the dioxin-responsive strain C57BL/6 was used throughout the study. Ah-Receptor containing nuclear protein extracts were prepared from a thymic epithelial cell line (ET) according to standard procedures, and incubated with ^{32}P-radiolabelled putative DREs from the IL-2 promoter and different unlabelled competitor DNA sequences. The complex-containing solutions were separated on a polyacrylamide gel and autoradiographed. To determine the specifity of the shifted bands, anti-AhR antibodies were added as well, resulting in supershifts.

Fetal thymi were excised from embryos at day 15 of gestation, and kept *ex vivo* in whole organ cultures for up to six days in the presence or absence of 10 nM 2,3,7,8-TCDD dissolved in 1,4-dioxane. For *in vivo* experiments 6 to 8 week old mice were injected i.p. with 50µg 2,3,7,8-TCDD/kg body weight, and their thymocytes or splenocytes taken out 6 days later. RNA was prepared from the cells directly, and RT-PCR with IL-2 primers was performed. Alternatively, thymocytes or splenocytes of unexposed mice were cultivated with or without TCDD for four hours in 6-well culture plates coated with different amounts of anti-CD3 antibodies. The IL-2 mRNA content was likewise measured by RT-PCR. PCR-products were separated on polyacrylamide gels and autoradiographed. The resulting band intensities were scanned densitometrically. The house-keeping gene HPRT was measured for each sample in parallel and used as a standard to calibrate for the induction of IL-2. Results are expressed as the ratio IL-2/HPRT intensity of a single PCR.

Results and discussion

Band shift gel assays of the three mouse DRE consensus sequences identified in the IL-2 promoter were performed. As shown in Table 1 all three DREs (positioned at - 1261, -851, and - 815) could specifically bind to and „shift" nuclear extracts of an Ah-receptor containing thymus epithelial cell line, ET. The resulting complex could be detected by anti-Ah-receptor antibodies.

TABLE 1

	– TCDD	+TCDD[a]				
		^{32}P DRE[b]	unlabelled DREs	human DRE	nonspec. compet.	anti-AhR antibodies
DRE I[c] (-1270 to -1241)	no[d]	↑[d]	no	no	↑	↑↑
DRE II (-866 to -831)	no	↑	no	no	↑	↑↑
DRE III (-825 to -794)	no	↑	no	no	↑	↑↑

[a] ET cells were incubated with 10nM 2,3,7,8-TCDD and nuclear protein extracts were prepared. Extracts were incubated with one of the DNA-sequences and loaded onto a polyacrylamide gel.

[b] either ^{32}P-labelled DRE (I,II, or III), unlabelled DREs as specific competitor, the human consensus DRE12, or an unrelated DNA-sequence (NS4) was added to the nuclear protein extracts. In addition to the ^{32}P-labelled IL-2-DREs anti-AhR antibodies was added.

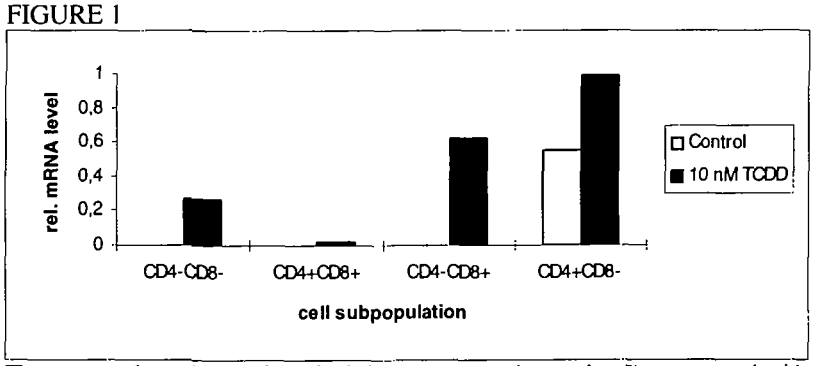
[c] DRE sequences from the 5´ region of the IL-2 promoter (see ref. 1) were synthesized and used for in the binding studies

[d] no= no band shift was detectable, ↑= an Ah-receptor band shift was detectable, ↑↑= detection of a supershift in the gel.

The presence of Ah-receptor binding DRE-sequences may be considered an important prerequisite for direct transcriptional activation via the TCDD-liganded Ah-receptor. Thus, the upregulation of IL-2 mRNA expression in fetal thymi[2] can be due to direct transcriptional activation. However, since most promoters are modular, and controlled differentially by more than one „element", the physiological significance of DREs in a given promoter for a given cell type and its differentiation stage has to be shown for each single case. Transcriptional activation *via the* Ah-R/TCDD complex was shown conclusively only for the CytP450 1A1 gene, albeit the Ah-receptor induces a battery of genes, all of which contain consensus DREs in their promoters. We are currently investigating whether the DREs in the IL-2 promoter are functional, i.e. whether they can control the transcription of reporter genes in transcription assays in appropriate cell lines (e.g. T-cells).

IL-2 was inducible in organ cultured fetal thymi[2]. The effects of TCDD on fetal thymi, i.e. acceleration of differentiation or skewing of the differentiation towards CD8-single positive, prospective cytotoxic T-cells, are mediated via the thymus stroma[3]. We sorted thymocytes from day 5 of fetal thymus organ cultures and found that the IL-2 increase was not due to stromal cells, but rather to both the very immature and the mature, prospective T-cell subpopulation (CD4-CD8- and CD4+CD8-, CD4-CD8+, respectively (see Fig 1). This fits well with data from the literature that IL-2 is produced in T-cells. Moreover, it shows that TCDD affects thymic development in more than one way. In this respect, the induction of IL-2 in the CD4-CD8- cells is quite interesting, because IL-2 is known to act as a differentiation cytokine on this developmental stage.

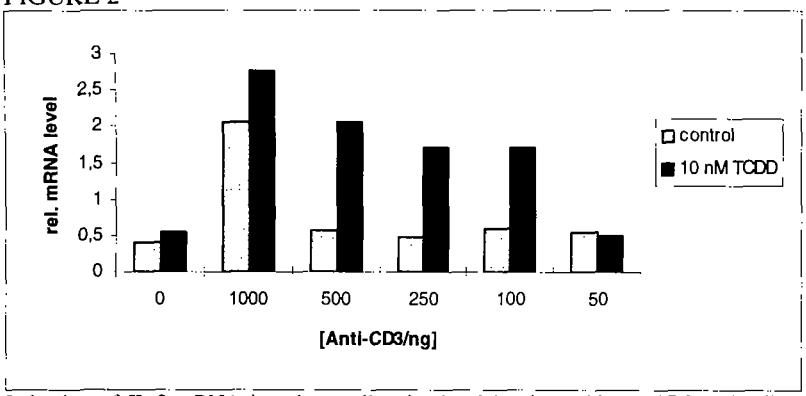
FIGURE 1



Thymocyte subpopulations from fetal thymus organ cultures (day 5) were sorted with the fluorescence activated cell sorter. RNA was prepared and RT-PCR was performed for IL-2 and hypoxanthinphopho-ribosyltransferase (a constitutively expressed housekeeping gene). Amplified products were scanned densitometrically and the ratio of IL-2 expression over HPRT-expression was calculated.

Our experiments showed (a) that the IL-2 promoter is in principle inducible by TCDD, and (b) that indeed in certain T-cell differentiation stages (i.e. in thymocytes) IL-2 is induced by TCDD. Moreover, in thymi of mice injected with a high dose of TCDD (50µg/kg) IL-2 mRNA was detectable after 6 days (data not shown). Since immunosuppression is a striking feature of TCDD-exposure in many animals, and IL-2 is a key cytokine in immune function, we tested whether IL-2 is modulated by TCDD also in peripheral T-cells. In physiological situtations IL-2 is produced by T-cells upon antigen activation of the T-cell receptor/CD3 complex, requiring the proper triggering by at least two signals simultaneously (antigen and CD28-ligand)[4]. Gross cross-reacting the CD3-receptors on the T-cell surface by high concentrations of anti-CD3 antibodies can bypass these two signals and stimulates T-cells to IL-2 production. We have stimulated spleen cells from C57BL/6 mice *in vitro* with anti-CD3 antibodies and TCDD. As shown in Fig. 2, TCDD acts synergistically with anti-CD3 to increase IL-2 mRNA. The basal expression of mRNA remains unaffected by TCDD without co-stimulation by anti-CD3 or at very low concentrations. At high concentrations the TCDD-induced increase is almost undistinguishable from the anti-CD3 effect. However, within a certain concentration range, TCDD causes an induction of IL-2 mRNA similar to that achievable by high anti-CD3 concentration.

FIGURE 2



Induction of IL-2 mRNA in spleen cells stimulated in vitro with ant-CD3 antibodies and 10nM 2,3,7,8-TCDD. Spleen cells were incubated with the indicated concentrations for four hours. RNA was prepared and RT-PCR was performed for IL-2 and hypoxanthinphophoribosyltransferase. Amplified products were scanned densitometrically and the ratio of IL-2 expression over HPRT-expression was calculated.

It was reported that in some antigen-activated T_H-cell clones IL-2 was not inducible, and that *in vivo* the antigen-specific T-cell response is suppressed by TCDD[5,6]. It was speculated that T-cells may lack a factor necessary for transcriptional activation of IL-2 via the Ah-receptor or, respectively, contains suppressor factor(s). The signal of anti-CD3 may provide such a factor. IL-2 transcription is triggered after the joining of two signalling cascades ending in upregulation of c-jun and c-fos, respecitively. Both proteins then form the ubiquitous transcription factor AP-1. We have found c-jun mRNA in thymocytes to be upmodulated by TCDD. Thus, it is tempting to speculate that TCDD interferes with the this one branch of the signalling cascade and by upregulating c-jun complements the first signal triggered by anti-CD3. Apart from the IL-2 gene, this pathway may be important also for some of the many other genes known to be modulated by TCDD. Alternatively, TCDD may trigger transcription of the IL-2 gene directly via Ah-receptor binding to the IL-2 DREs. We are currently investigating both possibilities.

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