Assessment of immune cell functions in diluted whole blood samples from industrial workers after exposure to 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD): Reduced antigen specific T-cell responses in diluted whole blood cultures but not in isolated mononuclear cells.

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1. Introduction

Although many studies on the toxic effects of halogenated hydrocarbons and especially on 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) have been performed little is known of the interference of TCDD with immune functions in humans. In experimental animals the impairment of the immunesystem appears to be a very sensitive means for the assessment of even "low dose" TCDD toxicity [1,2]). The influence of TCDD exposure on the human immune system, however, seems not so clear. Neubert et al. [3]) did not find significant changes in the proliferation capacities of lymphocytes derived from persons with moderately increased body burdens of TCDD whereas in a recent paper Tonn et al. [4]) report a decreased responder cell function in allogeneically stimulated peripheral blood lymphocytes derived from industrial workers who had been exposed 20 years before to considerable concentrations of TCDD. Other parameters characterizing the immune system like lymphocyte surface markers or the capacity of lymphocytes to respond to T-cell mitogens, however, were no altered in comparison to lymphocytes of control persons who had not been exposed to TCDD.

The objective of our study was a comparative analysis of phenotype and function of peripheral blood leukocytes from two cohorts of industrial workers of chemical plants, one but not the other of which had been exposed occupationally to high concentrations of TCDD.

2. Material and Methods, Volunteers

Volunteers: TCDD-exposed group

All men who have been working for at least 7 years in the trichlorophenolic acid production unit of one single chemical plant in Hamburg and who were still living in the Hamburg area were asked to volunteer in this study. Twenty one out of these 28 persons gave their informed consent to participate in this study. Two out of the 21 volunteers suffered from diabetes mellitus and thus were excluded from our statistical evaluation. The remaining 19 persons of the TCDD-exposed group had a mean age of 55.4 (range 40.8 - 69.3, median 57.7) years. Their actual TCDD burden varied from 33.6 ng/kg to 2252 ng/kg and the concentration of TCDD exposure at the time when they left the company ($TCCD_{back}$) was estimated assuming a half life time of 7.1 years [5]) and is depicted in table 1 together with the values of toxic equivalents.

Volunteers: Control group

All volunteers of the control group were recruited from another chemical plant in Hamburg where no

occupational TCDD exposure could be expected. The determinations of both TCDD and β-HCH in the blood of these volunteers confirmed that the burden of TCDD and β-HCH was within the range of unexposed people in Germany. Due to the age frequencies in the TCDD-exposed group the 28 male workers of the control group were selected by frequency-matching.

	TCDD-exposed group						control group					
	mean	median	Std.	min.	max.	N	mean	median	Std.	min.	max.	N
Age years	55.5	57.7	7.3	40.8	69.3	19	53.9	54.0	6.9	43.0	66.0	28
TCDD ng/kg	385.6	115.8	555.7	33.6	2252.0	19	3.9	3.9	0.5	2.9	6.0	28
$TCDD_{back}$ ng/kg	585.9	219.0	727.7	60.8	2714.6	19	11.3	11.2	1.5	6.2	17.3	28
toxic equivalents	512.4	252.3	640.7	82.3	2732.9	19	15.4	15.4	1.7	12.0	22.8	28
β-HCH µg/l	40.8	17.9	54.2	2.2	192.0	14	0.6	0.4	0.5	0.2	1.4	5

Table 1 Characteristics of the TCDD-exposed group and of the control group

Immunological investigations.

Preparation of cells: For our immunological investigations 20 ml of peripheral blood were drawn by venipuncture into a sterile tube containing 20 U heparin /ml blood. One ml of blood was used for diluted whole blood cultures. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation. The polymorphonuclear granulocytes (PMN) containing "erythrocyte sediments" of the Ficoll-Hypaque centrifugation were mixed with two volumes of polyvinylalcohol and after sedimentation for 20 min the PMN enriched supernatant was collected and freed from contaminating erythrocytes by hypotonic lysis.

In vitro stimulation of cytokine release: In order to assess the capacity of lymphocytes and monocytes to release cytokines in response to appropriate stimuli we decided to culture the cells in parallel in two different culture systems. (i) Standard cultures with isolated PBMC in RPMI 1640 medium supplemented with stimulus plus 5 % FCS in tissue culture microtiter plates (0.2 Mio PBMC in 200 µl per well) and (ii) cultures of 1:12 diluted whole blood (50 µl blood plus 550 µl RPMI 1640 inclusive stimulus added) which could reflect perhaps better influences of the *in vivo* milieu on the leukocyte functions.

Using these culture systems the following cytokine-inducing stimuli were applied:

T-cell mitogens: phytohemagglutinin (PHA, 5µg/ml), anti-CD3 monoclonal antibodies (10 ng/ml)

Antigens : tetanus-toxoid (TT, 6 LF/ml), purified protein derivative of tuberculin (PPD, 10 µg/ml)

Interferon-α (IFN-α) stimulators: Newcastle disease virus (NDV), inactivated Polio viruses types I-III

Supernatants of both culture systems were taken after 16-20 h for the determination of the monokines IFN-α and tumor necrosis factor α (TNF-α) and after 4 days of culture for the determination of the major T-cell lymphokine interferon-γ (IFN-γ). All cytokines were determined by ELISA.

Leukocyte phenotyping: In order to characterize lymphocyte subpopulations PBMC were stained with fluorochrome conjugated monoclonal antibodies and then analyzed by flowcytometry using a cytofluorograf system 50H (Ortho). The following surface antigens were assayed: CD3, CD4, CD8, CD19, CD16, CD57, CD45RA, CD45R0, HLA-DR.

Phagocyte functions: Isolated granulocytes (PMN) [0.2 Mio in 300 µl Hepes-buffered DMEM Medium] and 300 µl of 1:10 diluted whole blood were stimulated by phagocytic stimuli zymosan (0.1 mg/ml) and latex particles (ø 1 µm, 10^8/ml) and by the chemotactic peptide FMLP (1 µM) and

the release of activated oxygen species was traced as luminol mediated chemiluminescence for 25 min at 37°C using two six channel luminometers (Biolumat 9505, Berthold).
Statistics. In order to characterize the experimental results in the two cohorts we determined mean, standard deviation (Std.), median, minimal and maximal values of all measuring parameters. For the determination of statistically significant differences we performed the nonparametric Mann-Whitney U-Test and regarded differences with p-values less than 0.05 as significant.

3. Results

Cytokine release

With regard to the polyclonally stimulated releases of IFN-γ and TNF-α by PHA and by anti-CD3 monoclonal antibodies no significant differences of the mean values of the two cohorts were observed in either culture system (isolated PBMC or diluted blood cultures).
In contrast, the tetanus-toxoid (TT)-induced IFN-γ release in diluted whole blood cultures of the TCDD-exposed group was less than in the control group ($p<0.05$) whereas the TT-induced IFN-γ release in PBMC of the TCDD-exposed was higher than in the control group. The ratio of TT-induced IFN-γ in blood versus PBMC culture is therefore again significantly lower in cultures from TCDD-exposed workers than from control workers ($p<0.03$). A similar dissociation of cytokine responses in PBMC versus diluted blood cultures was seen in PPD-induced TNF-α release: Whereas in PBMC cultures of the TCDD-exposed group the PPD-induced TNF-α release was slightly higher than of the control group in diluted whole blood cultures the PPD-induced TNF-α release was significantly less in cultures of TCDD-exposed than of control workers ($p<0.05$) (cf. Table 2).
With respect to virus-induced (T-cell independent) IFN-α release we found no different Polio-induced IFN-α release in dilute whole blood cultures in both groups, however, Polio-induced IFN-α release from PBMC cultures as well as NDV-induced IFN-α release in both culture systems was lower in cultures of TCDD-exposed workers than in cultures of control workers but this decreased IFN-α release did not reach the level of statistical significance ($p=0.07$).

	TCDD-exposed group						control group						U Test
	mean	median	Std.	min.	max.	N	mean	median	Std.	min.	max.	N	P
TT-ind. IFN-γ, bl. [a]	182.6	78.0	238.4	10.0	713.0	12	423.4	233.0	472.3	26.0	1665.0	13	0.04
TT-ind.IFN-γ ratio blood/PBMC	0.84	0.23	1.26	0.01	3.20	12	1.84	1.24	1.56	0.11	4.82	13	0.025
PPD-ind. TNF-α, bl. [a]	162.5	109.5	185.2	39.0	616.0	8	277.6	206.5	223.8	77.0	1001.0	16	0.043
$CD8^+CD45R0^+$ Ly %	9.4	8.5	4.8	2.4	20.0	19	5.8	5.2	3.4	1.9	17.0	28	0.008
$CD8^{dim}CD57^+$ Ly %	24.7	25.0	8.4	12.0	45.0	19	19.0	19.5	8.5	6.4	46.0	28	0.013
$CD45RA^+$ Ly %	58.2	58.0	10.5	35.0	75.0	19	68.9	68.0	9.5	54.0	94.0	28	0.003
Basophils in blood %	0.90	1.0	0.74	0.00	2.00	19	0.68	0.00	1.54	0.00	8.00	28	0.044
Latex-ind. CL in PMN (rel. light units/ 20 min)	723.2	708.5	318.0	248.2	1495.8	19	445.1	380.2	256.7	200.0	1375.8	28	0.0008
FMLP-ind. CL blood (rel. light units/ 20 min)	074.3	946.0	619.0	175.0	2554.0	19	782.6	636.0	686.4	216.0	3779.0	28	0.0288

[a] Cytokine concentrations are given in pg/ml

Table 2 Summary of phenotypical and functional parameters of peripheral blood leukocytes which differ significantly in the TCDD-exposed group as compared to the control group

Phenotypic analysis

We performed a careful phenotypic analysis of the PBMC of both groups using single color as well as dual color labelling and found in both cohorts comparable proportions of CD3, CD4, and CD8 positive T-cells, of CD19 positive B-cells and of CD16 positive NK-cells. However, in lymphocytes of the TCDD-exposed group we found significantly increased proportions of cytotoxic "memory" T-cells ($CD8^+CD45R0^+$) ($p<0.009$) and on "activated" $CD8^{dim}CD57^+$ cells ($p<0.02$) and significantly less lymphocytes with "naive" phenotype ($CD45RA^+$) ($p<0.003$) as compared to lymphocytes of the control group. The evaluation of bloodsmears yielded a slight but significant increase of basophils ($p<0.05$) in the blood of TCDD-exposed donors.

Phagocyte chemiluminescence

Both phagocytic stimuli used (zymosan and latex particles) and the stimulation with the chemotactic peptide FMLP induced higher mean chemiluminescence (CL) activities in diluted blood and in isolated PMN from TCDD-exposed donors as compared to the CL-activities of phagocytes from control donors. These differences were highly significant in the case of latex-particles induced CL-activity in PMN ($p<0.0008$) and in FMLP-induced CL-activity in diluted whole blood ($p<0.03$).

4. Discussion and conclusion

One major result of our investigation is the finding that in TCDD-exposed donors the same immune cells which respond adequately with IFN-γ release to an antigenic stimulus (i.e. tetanus-toxoid) if stimulated as isolated PBMC show an impaired immune response in diluted whole blood. This finding was clearly seen with tetanus-toxoid as antigenic stimulus but with PPD-stimulation a similar effect was only found with respect to PPD-induced TNF-α release from monocytes. It was not at all seen in polyclonal stimulation with PHA. Therefore we propose that exposure to high doses of TCDD can partially impair in the "blood milieu" T-cell / monocyte interactions which are essential for antigen specific T-cell responses whereas isolated PBMC of the same donors appear functionally nearly unaffected. The striking increase, however, of T-cells with a phenotype pattern of cytotoxic memory cells in TCDD-exposed persons may relate to the decreased alloreactivity and the suppressive activity of PBMC from TCDD-exposed workers described by Tonn et al. [4]). Our finding that phagocytes of TCDD-exposed persons show a stronger release of reactive oxygen species may be due to *in vivo* preactivation of the unspecific defense functions of monocytes and granulocytes.

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5. References

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