Immune Cell Functions in Industrial Workers after Exposure to 2,3,7,8-Tetrachlorodibenzo-p-dioxin: Dissociation of Antigen-Specific T-Cell Responses in Cultures of Diluted Whole Blood and of Isolated Peripheral Blood Mononuclear Cells

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A comparative analysis was performed of the phenotype and function of peripheral blood leukocytes of two age-matched cohorts of industrial workers in chemical plants, one of which was exposed occupationally to high concentrations of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Median actual TCDD burdens were 116 ng/kg and 4 ng/kg, respectively. The phenotype analysis of peripheral blood mononuclear cells (PBMC) revealed no significant differences in the proportions of CD3, CD4, or CD8+ T lymphocytes, of CD16+ natural killer cells, and of CD19+ B lymphocytes. However, in PBMC of the TCDD-exposed workers, the proportion of CD8+ memory T cells (CD45R0+) was significantly higher, and that of lymphocytes with naive phenotype (CD45RA+) was significantly lower than in PBMC of the control group. Polyclonal and antigen-specific T-cell activation was assessed in parallel in isolated PBMC as well as in diluted whole blood cultures. In both culture systems the polyclonally stimulated cytokine release did not differ significantly between the two cohorts; however, we found a significantly reduced interferon γ release in diluted whole blood cultures but not in isolated PBMC cultures of the TCDD-exposed cohort when we performed an antigen-specific T-cell stimulation with tetanus-toxoid. Therefore, we propose that exposure of individuals to high doses of TCDD can partially impair in the “blood milieu” those T-cell/monocyte interactions that are essential for antigen-specific T-cell responses, whereas isolated PBMC of the same donors appear functionally less affected.—Environ Health Perspect 106(Suppl 2):701–705 (1998). http://ehpnet1.niehs.nih.gov/docs/1998/Suppl2/701-705ernstabstract.html

Key words: dioxin, diluted blood cultures, immune cell functions, interferon γ, occupational exposure, TCDD

Introduction

Many studies on the toxic effects of halogenated hydrocarbons and especially on 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) have been performed; however, little is known of the interference of TCDD with immune functions in humans. In experimental animals impairment of the immune system appears to be a sensitive means to assess even low-dose TCDD toxicity (1,2). In contrast, the influence of TCDD exposure on the human immune system is not as 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 decreased responder cell function in allogenically stimulated peripheral blood lymphocytes from industrial workers exposed 20 years before to considerable concentrations of TCDD. Other parameters characterizing the immune system such as lymphocyte surface markers or the capacity of lymphocytes to respond to T-cell mitogens, however, were not altered compared to lymphocytes of control persons not exposed to TCDD. Also, in a recent study on the effect of TCDD added in vitro to human peripheral blood mononuclear cells (PBMC), no phenotypic or proliferation changes were seen after polyclonal stimulation of isolated PBMC (5).

This study is a comparative analysis of phenotype and function of peripheral blood leukocytes of two cohorts of industrial workers in chemical plants, one of which was exposed occupationally to high concentrations of TCDD.

Materials and Methods

Volunteers

All men who worked at least 7 years in the trichlorophenolic acid production unit of a single chemical plant in Hamburg, Germany, and who were still living in the Hamburg area were asked to volunteer in this study. Of these 28 persons, 21 consented to participate in the study. Of the 21 volunteers, 2 suffered from diabetes mellitus and thus were excluded from our study. The remaining 19 persons in the TCDD-exposed group had a mean age of 55.4 years (range 40.8–69.3; median 57.7). At the time of the study all persons in the exposed group had left the chemical plant at least 10 years before (the time since their leaving the company ranged from 10 to 23 years; the median was 13 years). Their actual TCDD burdens varied from 33.6 to 2252 ng/kg blood fat and the concentration of TCDD exposure at the time they left the company (TCDDback) was estimated assuming a half-life of 7.1 years (6). This is depicted in Table 1 together with the toxic equivalents values. The TCDD analyses were performed as described earlier (7).
All volunteers in the control group were recruited from another chemical plant in Hamburg in which no occupational TCDD exposure was expected. Because of the age frequencies in the TCDD-exposed group, the 28 male workers of the control group were selected by frequency matching. The proportion of smokers was approximately the same in both groups—7 of 19 in the TCDD-exposed group (37%) vs 11 of 28 in the control group. In 5 of the control group volunteers chosen at random, TCDD concentrations in the blood were determined; their burdens of TCDD were within the range of the unexposed people in Germany. Characteristics of the TCDD-exposed group and the control group are given in Table 1.

### Immunologic Investigations

#### Preparation of Cells
For our immunologic investigations 20 ml of peripheral blood were drawn by venipuncture into a sterile tube containing 20 U heparin/ml blood. One milliliter of blood was used for diluted whole blood cultures. PBMC were isolated by Ficoll-Hypaque density gradient centrifugation. The polymorphonuclear granulocytes (PMNs) containing erythrocyte sediments of the Ficoll-Hypaque centrifugation were mixed with 2 vol 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
To assess the capability of lymphocytes and monocytes to release cytokines in response to appropriate stimuli, we cultured the cells in parallel in two different culture systems: a) standard cultures with isolated PBMC in RPMI 1640 medium supplemented with stimulus plus 5% fetal calf serum in tissue culture microtiter plates (0.2 Mio PBMC in 200 μl per well) and b) cultures of 1:12 diluted whole blood (50 μl blood plus 550 μl RPMI 1640 inclusive stimulus added), which perhaps better reflect influences of the in vivo milieu on the leukocyte functions.

Using these culture systems we applied the following cytokine-inducing stimuli.

1. T-cell mitogens: phytohemagglutinin (PHA, 5 μg/ml; Murex Diagnostika, Burgwedel, Germany), anti-CD3 monoclonal antibodies (10 ng/ml; clone 83; Coulter-Immunotech, Hamburg, Germany).
2. Antigens: tetanus–toxoid (TT; 6 LF/ml; provided by Dr. F. Reber, Behringwerke AG, Marburg, Germany), purified protein derivative of tuberculin (PPD; 10 μg/ml; Statens Serum Institute, Copenhagen, Denmark).
3. Interferon α (IFN-α) stimulators: Newcastle disease virus (NDV) provided by Dr. R. Zawatzky (Deutsches Krebsforschungszenrum, Heidelberg, Germany), inactivated polio viruses types I to III (Behringwerke AG, Marburg, Germany).

#### Leukocyte Phenotyping
To characterize lymphocyte subpopulations, we stained PBMC with fluorochrome-conjugated monoclonal antibodies (antibodies against human leukocyte antigen [HLA-DR], CD45RA, and CD57; provided by Dako (Hamburg, Germany); all other monoclonal antibodies used were from Becton Dickinson (Heidelberg, Germany). Staining with monoclonal antibodies was performed on whole blood according to the manufacturer’s instructions. As a control, PBMC were stained with antibodies against CD57, HLA-DR, CD45RA, and CD57 (1 μg/ml; Sigma, Deisenhofen, Germany). The following antibody combinations were used for the analysis:

- CD57/HLA-DR/CD45RA for the determination of cytotoxic T-cells
- CD57/HLA-DR/CD45RA for the determination of CD57+CD45RA+ T-cells
- CD57/HLA-DR/CD45RA for the determination of CD57+CD45RA− T-cells
- CD57/HLA-DR/CD45RA for the determination of CD57−CD45RA+ T-cells
- CD57/HLA-DR/CD45RA for the determination of CD57−CD45RA− T-cells

### 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). Because in vitro antigenic T-cell responses depend on the presence of antigen-specific memory T-cells, we included in the analysis antigen-stimulated effect cells only those individuals in both cohorts whose T-cells responded to the respective antigen in either culture system by releasing at least 150 pg/ml IFN-γ. Thus, 10 TT responders and 8 PPD responders in the TCDD-exposed group and 12 TT responders and 15 PPD responders in the control group were assessed for their antigen-specific T-cell responses. In contrast to the polyclonally stimulated IFN-γ release, the TT-induced IFN-γ release in diluted whole blood cultures of the TCDD-exposed group was less than that in the control group (p < 0.05), whereas the TT-induced IFN-γ release in

### Table 1. Age distribution and TCDD burden of the TCDD-exposed workers and of the control group.

| Age, years | Mean | Median | SD  | Minimum | Maximum | n   |
|-----------|------|--------|-----|---------|---------|-----|
| TCDD group | 55.5 | 57.7   | 7.3 | 40.8    | 68.3    | 19  |
| Control group | 53.9 | 54.0   | 8.9 | 43.0    | 66.0    | 29  |
| TCDD, ng/kg | 385.6 | 115.8  | 555.7 | 33.6 | 2252.0 | 19  |
| Control group | 4.0 | 3.4    | 1.3 | 2.9     | 6.0     | 5   |
| TCDD_{eq}, ng/kg | 585.9 | 219.0  | 727.7 | 60.8 | 2714.6 | 19  |
| Control group | 11.8 | 11.9   | 4.0 | 6.2     | 17.3    | 5   |
| Toxic equivalents, ng/kg | 512.4 | 252.3  | 640.7 | 82.3 | 2732.9 | 19  |
| Control group | 15.4 | 13.7   | 4.5 | 12.0    | 22.8    | 5   |
PBMC of the TCDD-exposed group was higher than that in the control group. The ratio of TT-induced IFN-γ in blood compared to that in PBMC culture was 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 compared to that in diluted blood cultures was seen in PPD-induced (and T-cell-dependent) TNF-α release. In PBMC cultures of the TCDD-exposed group, PPD-induced TNF-α release was slightly higher than that of the control group whereas in diluted whole blood cultures the PPD-induced TNF-α release was significantly lower in cultures of TCDD-exposed workers than of control workers (p < 0.05) (Table 2).

With respect to virus-induced (T-cell independent) IFN-α release, we found no different polio-induced IFN-α release in diluted whole blood cultures in either the TCDD-exposed group or the control group. 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. This decreased IFN-α release did not reach the level of statistical significance (p = 0.07).

### Phagocyte Chemiluminescence

Both phagocytic stimuli that were used (zymosan and latex particles) and stimulation with the chemotactic peptide FMLP induced higher mean chemiluminescence (CL) activities in diluted blood and in isolated PMN from TCDD-exposed donors than in control donors. These differences were highly significant in the case of latex particle-induced CL activity in PMN (p < 0.0008) and in FMLP-induced CL activity in diluted whole blood (p < 0.03) (Table 2).

### Phenotypic Analysis

We performed a careful phenotypic analysis of PBMC in both groups using single color as well as dual-color labeling and found in both cohorts comparable proportions of CD3-, CD4-, and CD8+ T cells, of CD19+ B cells, and of CD16+ natural killer (NK) cells. Also, the CD4/CD8 ratio of lymphocytes was comparable in both cohorts. However, in lymphocytes of the TCDD-exposed group, we found significantly increased proportions of cytotoxic memory T-cells (CD8+CD45RO+) (p < 0.009) and activated CD8dimCD57+ cells (p < 0.02) and significantly fewer lymphocytes with naive phenotype (CD45RA+) (p < 0.003) compared to lymphocytes of the control group. The CD4/CD8 ratio within the T lymphocytes with memory phenotype (CD45RO), therefore, appeared to be significantly reduced in the TCDD-exposed cohort (p < 0.002). We also found a slightly lower proportion of HLA-DR+ monocytes in the TCDD-exposed group than in the control cohort (p < 0.05) (Table 3).

### Correlations with TCDD Burden

Analysis of correlations between the TCDD burden and the phenotypical and functional parameters revealed inverse correlations of the TCDD burden with IFN-α release stimulated by NDV in both culture systems as well as with the proportion of naive lymphocytes. On the other hand, the proportion of activated T lymphocytes and the FMLP-induced release of reactive oxygen species appeared to increase with increased TCDD burden (Table 4).

### Discussion and Conclusion

Our study included only those industrial workers in both cohorts (workers occupationally exposed to TCDD and those not exposed to TCDD) not suffering from carcinoma or obvious autoimmune diseases such as diabetes mellitus. In healthy individuals not exposed to TCDD, most cellular immunologic parameters that could be tested in vitro presented with considerable interindividual scattering. Therefore, those immune parameters that differ significantly between the two groups might be of biologic importance. One major result of our investigation is the finding that in TCDD-exposed donors the same immune cells that respond adequately with IFN-γ release to an antigenic stimulus (i.e., TT) when stimulated as isolated PBMC show impaired immune responses in diluted whole blood. This finding was clearly observed with TT as the antigenic stimulus, but with PPD stimulation a similar effect was found only with respect to PPD-induced (and T-memory-lymphocyte-dependent) TNF-α release from monocytes. This effect was not observed in polyclonal stimulation with PHA. Other authors also observed the

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### Table 2. Functions of peripheral blood leukocytes including stimulated release of cytokines from T lymphocytes (IFN-γ) and from monocytes (TNF-α, IFN-α) and phagocyte activation (chemiluminescence activity).

|                          | Mean | Median | SD   | Minimum | Maximum | n   | p value* |
|--------------------------|------|--------|------|---------|---------|-----|----------|
| **TT-induced IFN-γ, pg/ml, blood** |      |        |      |         |         |     |          |
| TCD2 group               | 197  | 68     | 261  | 10      | 713     | 10  | 0.05     |
| Control group            | 449  | 246    | 484  | 26      | 1685    | 12  |          |
| **TT-induced IFN-γ, pg/ml PBMC** |      |        |      |         |         |     |          |
| TCD2 group               | 508  | 297    | 372  | 188     | 1127    | 10  | 0.08     |
| Control group            | 375  | 187    | 533  | 39      | 1874    | 12  |          |
| **TT-induced IFN-γ/blood/PBMC ratio** |      |        |      |         |         |     |          |
| TCD2 group               | 0.74 | 0.23   | 1.22 | 0.02    | 3.20    | 10  | 0.021    |
| Control group            | 1.93 | 1.51   | 1.80 | 0.11    | 4.82    | 12  |          |
| **PHA-induced IFN-γ, pg/ml blood** |      |        |      |         |         |     |          |
| TCD2 group               | 7.86 | 5.37   | 9.05 | 0.76    | 39.09   | 19  | NS       |
| Control group            | 10.41| 5.01   | 15.06| 0.59    | 78.33   | 28  |          |
| **PPD-induced TNF-α, pg/ml blood** |      |        |      |         |         |     |          |
| TCD2 group               | 163  | 110    | 185  | 39      | 616     | 8   | 0.020    |
| Control group            | 291  | 213    | 225  | 86      | 1001    | 15  |          |
| **Polio-induced IFN-α, pg/ml PBMC** |      |        |      |         |         |     |          |
| TCD2 group               | 80   | 31     | 118  | 2       | 493     | 19  | 0.07     |
| Control group            | 140  | 84     | 148  | 2       | 624     | 26  |          |
| **Latex-induced CL, relative light units/20 ft PMN** |      |        |      |         |         |     |          |
| TCD2 group               | 723  | 709    | 318  | 248     | 1496    | 19  | 0.0008   |
| Control group            | 445  | 380    | 257  | 200     | 1376    | 28  |          |
| **FMLP-induced CL, relative light units/20 ft blood** |      |        |      |         |         |     |          |
| TCD2 group               | 1074 | 946    | 619  | 175     | 2564    | 19  | 0.0288   |
| Control group            | 783  | 636    | 666  | 216     | 3779    | 28  |          |

NS, not significant. *Mann-Whitney U-test.
absence of TCDD-induced changes in T-cell proliferation in polyclonally stimulated, isolated PBMC of TCDD-exposed persons or in in vivo TCDD-treated PBMC of healthy donors (4,5). Therefore, we suggest that exposure of individuals to high doses of TCDD can partially impair in the blood milieu those T-cell/monocyte interactions that are essential for antigen-specific T-cell responses; isolated PBMC in the same donors appear functionally less affected. Our results indicate that the reduced IFN-γ release in antigen-driven but not in polyclonally induced T-cell stimulation seen in the TCDD-exposed cohort is not due to a TCDD-mediated impairment of the IFN-γ production machinery within T cells per se but relates rather to regulatory elements involved in antigen-driven T-cell activation such as the function and phenotype of accessory cells and/or soluble stimulatory or inhibitory factors. In this context it is interesting to note that the TCDD burden is inversely related to the NDV-induced IFN-α release in both culture systems (Table 4). As shown earlier (8), the stimulation of PBMC with NDV leads to the release of IFN-α nearly exclusively in a small subpopulation of human monocytes, namely those monocytes lacking the Fcγ receptor I (high-affinity Fc receptor for IgE) (CD64). Another characteristic of this monocyte subpopulation is its function as professional antigen-presenting cells and their high accessory capacity for the interaction with T lymphocytes, which is related to the high expression of MHC I (major histocompatibility complex class I) and MHC II (HLA-DR) as well as to the fact that CD64+ monocytes are more resistant to killing by antigen-activated T cells than CD64+ monocytes (9). In the present study we did not determine the proportion of CD64+ monocytes, which normally amounts to less than 5 to 8% of all monocytes; also, we did not try to enrich this subpopulation by cell sorting for distinct functional studies. However, because we have measured the NDV-induced IFN-α release, which appears to be restricted to the function of CD64+ monocytes, it is tempting to speculate that the reduced NDV-induced IFN-α release observed in the cultures of the TCDD-exposed group indicates the reduced function of professional antigen-presenting cells in peripheral blood. The lower HLA-DR expression in monocytes of the TCDD-group (Table 3) supports this idea. In addition the striking increase of T cells with phenotype patterns of activated cytotoxic memory cells, which confirms data from other studies (10) and in turn is associated with reduced proportions of naive (CD45RA+) T lymphocytes in TCDD-exposed persons may relate to the decreased alloreactivity and the suppressive activity of PBMC from TCDD-exposed workers, as 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 in the course of TCDD-promoted inflammatory reactions (11,12).

**Table 3. Phenotypical characterization of PBMC.**

|                            | Mean  | Median | SD   | n   | p value*  |
|---------------------------|-------|--------|------|-----|-----------|
| Naive lymphocytes, % CD45RA+ |       |        |      |     |           |
| TCDD group                | 58.3  | 58.0   | 10.5 | 19  | 0.0025    |
| Control group             | 68.9  | 68.0   | 8.5  | 28  |           |
| CD8 memory cells, % CD8+CD45RA+ |       |        |      |     |           |
| TCDD group                | 9.4   | 8.5    | 4.8  | 19  | 0.0087    |
| Control group             | 5.8   | 5.3    | 3.4  | 28  |           |
| CD4/CD8 ratio             | 1.73  | 1.42   | 0.88 | 19  | NS        |
| TCDD group                | 2.15  | 1.68   | 1.54 | 28  |           |
| Control group             |       |        |      |     |           |
| CD4 memory/CD8 memory ratio |       |        |      |     |           |
| TCDD group                | 2.11  | 1.50   | 1.51 | 19  | 0.0014    |
| Control group             | 3.52  | 3.03   | 1.93 | 28  |           |
| Activated CD8/null cells, % CD8+CD64+ |       |        |      |     |           |
| TCDD group                | 24.7  | 25.0   | 8.4  | 19  | 0.0129    |
| Control group             | 19.0  | 19.5   | 8.5  | 28  |           |
| % HLA-DR+ monocytes       | 85.3  | 85.0   | 7.4  | 19  | 0.05      |
| TCDD group                | 89.0  | 91.5   | 8.3  | 28  |           |
| Control group             |       |        |      |     |           |

* Mann-Whitney U-test.

**Table 4. Correlations with TCDD burden.**

|                         | Correlation with TCDD Spearman rank correlation coefficient, r | p value |
|-------------------------|---------------------------------------------------------------|---------|
| NDV-induced IFN-α blood | -0.59                                                         | 0.008   |
| NDV-induced IFN-α PBMC  | -0.58                                                         | 0.009   |
| Activated T lymphocytes, % CD3+HLA-DR+ | 0.43                                                      | 0.063   |
| Naive lymphocytes, % CD45RA+ | -0.46                                                        | 0.025   |
| Activated CD8/null cells, % CD8+CD64+ | 0.40                                                        | 0.055   |
| Erythrocytes/μl blood | -0.40                                                         | 0.053   |
| FMLP-induced CN blood  | 0.52                                                          | 0.01    |

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