OX-47 Expression as a Measure of Dioxin Exposure

Jasvant S. Badesha, Bojan Flaks and Ian A. Stenhouse

Department of Clinical Veterinary Science, Faculty of Medicine, University of Bristol, Langford House, Langford, North Somerset BS48 5DU, United Kingdom

Introduction

The toxic chlorinated dibenzo-*p*-dioxins and structurally related compounds are potent inducers of microsomal enzymes, carcinogens and immunotoxins, posing a serious environmental threat to health (1,2), the young being particularly susceptible (3). There are no satisfactory methods currently available that will estimate the level of human exposure to these chemicals. Measurement of levels in tissues such as fat and liver may of some use in establishing exposure but the required procedures are both impractical (owing to the invasive nature of sampling) and very expensive (GC/MS analysis). In addition, such procedures may be of little value in establishing the causation of disease. A simple biological indicator of dioxin exposure that does not involve sampling of body tissue is therefore highly desirable.

We have found that the expression of a functional cellular protein, OX-47, is suppressed by dioxins. The OX-47 molecule, which is normally expressed by a variety of immune cells undergoing activation, was first produced following immunization of mice with rat activated T cells (4). It is recognized by the MRC OX-47 monoclonal antibody (mAb) and can be detected by simple procedures using fluorescence-conjugated mAb.

OX-47 has a broad expression pattern in both haematopoietic and non-haematopoietic tissues in a number of species and is up-regulated upon activation. In the human, M6 (human OX-47) is expressed weakly on resting leukocytes but is strongly up-regulated on activated lymphocytes and monocytes, and is expressed on all haematopoietic cell lines (6). In the rat OX-47 is present at low levels on thymocytes, bone marrow cells and resting lymphocytes, and is markedly increased following activation of B and T cells. It is also expressed on a variety of epithelia, endothelial cells of brain capillaries, muscle and nerve cells, perineural cells, choroid plexus and gonads (7, 8). In the mouse, basignin (mouse OX-47) is expressed on kidneys, spleens, small intestine, liver and small amounts in testis and brain (8). In the chicken, HT7 (chicken OX-47) is expressed on brain endothelial cells, choroid plexus, the retinal pigment layer, epithelial cells of kidney tubules and on erythrocytes(9).

The procedure to detect these molecules on activated lymphoid cells is rapid, simple and inexpensive. We report here the effect of 2,3,7,8-TCDD, the most toxic of the chlorinated *dibenzo-p-dioxins* on the expression of OX-47 in rat splenic and peripheral blood mononuclear

cells. In addition, we have studied the effect of a mixture of two other dioxins, 1,3,6,8-TCDD and 1,3,7,9-TCDD, which have hitherto been considered to be non-toxic: currently they have a Toxic Equivalence Factor (TEF) of zero. They are produced as byproducts of 2,4-dichlorophenol and 2,4,5-trichlorophenol manufacture, and may be present at quite high levels in process waste and subsequently in the environment.

Materials and Methods

Reagents

The PCDDs studied were 2,3,7,8-TCDD (ex Diverse Analyticals, Manchester, England) and a 2.5:1 mixture of 1,3,6,8-TCDD and 1,3,7,9-TCDD (a gift from Dr S. Safe), of >99% purity. The 1,3,6,8-TCDD/1,3,7,9-TCDD mixture was analyzed by GC/MS and the 2,3,7,8-TCDD content was found to be <0.001%, the only other congeners present being were some non-2,3,7,8-containing PeCDD congeners at a total content of 0.4%). The compunds were dissolved in analytical grade acetone and incorporated with powdered Oxoid 41B diet.

Treatment of animals

Inbred female rats of the Leeds strain were maintained under standard conditions of lighting (7 a.m. to 7 p.m.) and temperature (23°C). They received water and Oxoid 41B diet *ad libitum*. A total of 60 animals of about 23 days of age were divided into 10 groups and each group fed the appropriate diet for 30 days. Three groups of rats were given 2,3,7,8-TCDD at total doses of 0.3, 3 and 30 μg/kg body wt. respectively and five groups were given the 1,3,6,7-TCDD/1,3,7,8-TCDD mixture at total doses of 0.3, 3, 30, 300 and 3000 μg/kg body wt. respectively. In addition, two untreated control groups were fed a normal diet. The rats were weighed weekly and were sacrificed on day 3 following the end of the treatment period.

Immunotoxicity assays

The animals were stunned and killed by cervical dislocation. Splenic cell suspensions were made in RPMI 1640 medium supplemented with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol (2-ME), and 100 i.u./ml penicillin.

Flow cytometry was employed for the measurement of OX-47 expression, and the determination of $CD4^{+}/CD8^{+}$ splenic cell ratios. Non-activated splenic cells and splenic cells activated for two hours with Concanavilin A (Con A) (2μg/ml) and lipopolysaccharide (LPS) (100μg/ml) were assessed for the expression of OX-47 and for their CD4/CD8 ratio by flow cytometry, using the appropriate monoclonal antibodies (Serotec, UK), by a modification of a previously described method (10). Briefly, 1×10^{6} chilled cells in 50μl of RPMI 1640 medium with 1% normal rat serum and 0.2% azide (complete medium) were added to 50μl medium or to 50μl optimally diluted monoclonal antibody (W3/25, OX8 and OX-47 monoclonals). They were incubated for 2 hours on ice and washed four times in complete medium before resuspension in 100μl of medium containing flourescein-labelled goat anti-mouse immunoglobulin G (IgG) at the optimal dilution of 1/2000. The cells were further incubated on ice for 1 hour, washed once and measured for green flourescence using an EPICS-CS flow cytometer (Coulter, Luton UK). Results were expressed as a percentage of the total number of cells counted.

Results and Discussion

At autopsy there was no evidence of any gross treatment-related effects at any of the doses used. The effects of PCDDs on growth, the immune system indicator (CD4/CD8 ratio) and on the expression of the OX-47 antigen are shown in Table 1.

Table 1. Toxic and immunotoxic effects of Dioxins on female Leeds rats.

	TOTAL DOSE OF DIOXIN, µg/kg B.Wt					
	0.0	0.3	3.0	30.0	300.0	3000.0
Congeners	**Body weight (g)**					
2,3,7,8-TCDD	109 ± 4	99 ± 5 (P=0.048)	96 ± 6 (P=0.004)	88 ± 7 (P=0.003)	ND	ND
1,3,6,8-TCDD / 1,3,7,9-TCDD	109 ± 4	115 ± 10 (NS)	112 ± 7 (NS)	110 ± 5 (NS)	104 ± 9 (NS)	95 ± 6 (P=0.002)
Congeners	**CD4/CD8 ratio in spleen**					
2,3,7,8-TCDD	1.35 ± 01	1.23 ± 0.06 (NS)	1.02 ± 0.10 (P=0.041)	0.96 ± 0.08 (P=0.025)	(ND)	(ND)
1,3,6,8-TCDD / 1,3,7,9-TCDD	1.35 ± 04	1.33 ± 0.05 (NS)	1. 30 ± 0.10 (NS)	1.23 ± 0.12 (NS)	1.41 ± 0.01 (P=0.048)	1.45 ±0.03 (P=0.036)
Congeners	**Percentage of Cells Expressing OX-47**					
2,3,7,8-TCDD	97 ± 2	68 ± 2 (P=0.008)	43 ± 3 (P=0.001)	7 ± 1 (P=0.0001)	ND	N.D.
1,3,6,8-TCDD / 1,3,7,9-TCDD	98 ± 5	96 ± 2 (NS)	80 ± 5 (P=0.008)	71 ± 2 (P=0.008)	45 ± 3 (P=0.001)	36 ± 2 (P=0.001)

P = degree of significance. NS = not significant when compared with corresponding control ND = not done..

2,3,7,8-TCDD at the 3 µg/kg body wt. dose appears to significantly reduce the ration of T helper cells (T_H) to cytotoxic T cells (T_C). In contrast, the high doses of the 1,3,6,8-TCDD/1,3,7,8-TCDD mixture produced a significant increase in the CD4/CD8 ratio, suggesting that the mixture significantly affects T_C rather than T_H subsets. Most importantly, the results suggest that exposure of rats to 0.3 - 30 µg/kg body weight of 2,3,7,8-TCDD can suppress OX-47 expression by more than 30% - 90% in splenic and peripheral blood mononuclear cells. A mixture of 1,3,6,8-TCDD and 1,3,7,9-TCDD was also able to affect OX-47 expression similarly. However, in order to obtain the same level of inhibition as induced by 2,3,7,8-TCDD, a 100-fold greater amount of the mixture was required. Preliminary unpublished results obtained in this laboratory have indicated that similar suppression of OX-47 occurs in activated peripheral blood mononuclear cells in dioxin-treated animals, and also that immunotoxic polycyclic aromatic hydrocarbons (PAHs) do not affect the expression of OX-47.

Although the cellular role of OX-47 has not been fully established, human OX-47 on tumour cells is thought to bind an unknown ligand on fibroblasts, stimulating collagenase production, and on other extracellular matrix metalloproteinases (11). An adhesion role for the fowl homologue is also proposed, since the 5All mAb inhibits neural extensions of retinal glial cells and reduces retinal cell reaggregation *in vitro* (12). The presence of IgSF domains in the OX-47 antigen suggests that it may react with other cell surface molecules or soluble factors such as cytokines (13). Seulberger's group (9) have suggested that the avian homologue, HT7, is

involved in cell surface recognition at the blood-brain barrier.

The wide tissue distribution of OX-47, indicating that it has an important physiological role, could account for the extreme toxicity of dioxins. The procedure described here may be adopted for measurement of OX-47 in peripheral blood mononuclear leukocytes and may prove a useful technique for the estimation of the severity of exposure of humans to dioxins.

The results obtained here also may have implications with regard to TEQ values in certain situations. The 1,3,6,8-TCDD/1,3,7,9-TCDD mixture appears to be about 100-fold less toxic than 2,3,7,8-TCDD, based on the findings presented here. This would indicate that the 1,3,6,8-TCDD and 1,3,7,9-TCDD congeners should have a Toxic Equivalence Factor (TEF) of 0.01. (In fact, this was the value assigned to the non-2,3,7,8-containing TCDD congeners under the old EPA scheme.) The implication of this for the TEQ calculated for the usual combustion pattern is minor since, although the 1,3,6,8- and 1,3,7,9-congeners are the major TCDD congeners, they are usually present at only about a factor of ten greater than the concentration of the 2,3,7,8-TCDD and hence using a TEF of 0.01 would only increase the TEQ by up to 10%. However, if the dioxin contamination originates from chlorophenol production or waste, then the 1,3,6,8-/1,3,7,9-congeners may be present at levels that are several hundred times that of 2,3,7,8-TCDD. In these circumstances, applying a TEF of 0.01 to their concentrations may increase the resultant TEQ by up to a factor of five, which might change both the health implications and remedial action required to a significant degree.

Note This method of detecting dioxin exposure with OX-47 and its equivalents is the subject of a patent application.

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