Effects of 2,3,7,8 -Tetrachlorodibenzo-p-dioxin on a murine model of a lupus-like nephritis

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1. Introduction.

The ubiquitous xenobiotic, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is one of the most potent immunosuppressive agents known, eliciting thymic atrophy at μg/kg doses in experimental animals, and suppressing primary immune responses at even lower doses[1] . TCDD has also been observed to have both estrogenic and anti-estrogenic properties in different systems[2]. We and others have demonstrated that both the kinetics of thymic atrophy induction and reductions in lymphocyte stem cell targets associated with this atrophy are remarkably similar[3,4] although these effects are mediated through distinctly different receptors[5]. We had previously suggested that since (1) thymic atrophy is associated with autoimmune diseases in neonatal mouse models, and (2)estrogens are associated with autoimmune disease, it is possible that TCDD, and related compounds could also be associated with autoimmunity[6].

There has been suggestive data that autoimune processes and/or hyperimmunity in humans might be affected by exposure to TCDD and related AhR binding compounds. In a case control study of exposed workers, antinuclear antibodies and circulating immune complexes were detected at a significantly elevated level [7]. Among US Air Force personnel determined to be exposed to TCDD-containing herbicides, an association has been established for diabetes and exposure[8]. Much pathogenesis of various forms of diabetes can be demonstrated to have autoimmune components. Hyperimmune responses (increased responses to mitogens, tuberculin, and/or tetanus toxoid) have been reported in individuals who consumed rice oil contaminated with compounds that activate the AhR[9] and in offspring of TCDD exposed Rhesus monkeys[10].

Female, but not male, F1 progeny of NZB and SWR (SNF1) mice develop a fatal immune complex glomerulonephritis, similar to that seen in the human autoimmune disease systemic lupus erythematosus (SLE), prior to one year of age, with pathogenesis apparent by 6 months. Immunoglobulin deposited in the nephritic kidneys bears an idiotype, Id^{LN} F1, and production of Id^{LN} F1 IgG in serum correlates with disease onset[11]. Evidence has accumulated supporting the role of CD4+Id^{LN} F1 reactive T-cells in causing the disease in the female mice[12].

We have recently demonstrated that we can accelerate or potentiate disease in male SNF1 mice by administering estradiol valerate in an oil depot once every four weeks. Disease acceleration or potentiation occurs at doses as low as 1 mg/kg. Appearance of idiotype specific deposits in the kidneys of male mice can be seen as early as two weeks after the third dose of estradiol (when the male mice are only 4-4.5 months old). After 5 such doses(when the mice are only 6-7 months old) some of the control animals have developed such deposits, but the frequency of these is only

30-50% of that seen in the treated animals. While deposition is not identical with disease, proteinurea has also been observed in many of the mice manifesting these deposits.

Preliminary studies have been initiated with male SNF-1 mice utilizing exposure to TCDD, in doses ranging from 5 to 80 μg/kg, once every four weeks starting at 7-8 weeks of age. We have also administered a single dose of 80 μg/kg to a 14 day old pregnant SWR female, and examined the consequences of exposure on 18 week old perinatally exposed SNF-1 offspring. In our model, our data thus far suggests that TCDD potentiates or accelerates autoimmune disease.

2. Material and Methods.

SNF-1 mice were obtained by breeding male NZB mice with female SWR/J mice, both from Jackson Laboratories (Bar Harbor, ME). Seven to eight week old male mice were injected intraperitoneally with either 80,30, or 5 μg/kg in olive oil. Control, randomized litter mates were injected simultaneously with olive oil alone. As both SWR and NZB mice have the Ah^{d} phenotype (less sensitive to TCDD), their progeny, the SNF1 mice are also resistant. Thus, while a single dose of 30 μg/kg in olive oil can cause 80-90% thymic atrophy in Ah^{bb} (sensitive) strains, the same dose in SNF-1 males causes statistically non-significant atrophy, and it is necessary to administer 80 μg/kg to achieve70-80% atrophy.

Mice were euthanized by CO_2 and 0.5 cc. of blood was obtained by cardiac puncture, for subsequent serum analysis. Thymus and spleens and, when observed, enlarged nodes were removed and single cell suspensions made by mechanical disaggregation. For spleen, red blood cells were lysed with buffered ammonium chloride. Cells were counted for number and viability. A T-lymphocyte-enriched population was obtained from the spleen cell suspension using a Biotex (Edmonton,Alberta) mouse T-cell column as per manufacturer's instructions. Liver lymphocytes were prepared by perfusing livers with PBS. Livers were excised, crushed through sterile stainless steel screens, and triturated cell suspensions were passed through 20 micron Nitex nylon mesh. RBCs were lysed as described, and lymphocytes were separated on a 40/70% Percoll step gradient.

Intestinal epithelial lymphocytes (IELs) were isolated by removing the small intestine, flushing it with cold buffered saline, and inverting the tissue so that the villi are exterior. Lymphocytes were removed by agitation for 30 min. at 37° in a digestion buffer containing 1mM DTT and 1mM EDTA. IELs were further enriched on a 40/70% percoll step gradient. Immunophenotyping demonstrated that more than 90% of the cells in the lymphocyte size/scatter gate were lymphocytes. Cells from these tissues were stained for three color flow cytometry analysis using FITC, PE, and biotin conjugated antibodies. Antibodies were either produced in our laboratories, or purchased from Pharmingen (San Diego, CA). Biotin was subsequently combined with streptavidin red 670 (Gibco/BRL, Gaithersburg, MD). Flow cytometric acquisition and analysis was carried out on a Becton Dickinson Facstar Plus as previously described[4]using the LySysII program.

The capsule was removed from both kidneys and one kidney from each animal was fixed in Zamboni's fixative, processed and embedded in a paraffin block. Sections were cut and stained with hematoxylin and eosin (H & E staining) using standard histologic methods. Analysis of an unselected group of treated and control kidneys was carried out in a blinded protocol, so that the reader did not know from which group the slides were derived. In each kidney 100 consecutive glomeruli were evaluated by light microscopy as a 40x objective was moved across the renal cortex. Each glomerulus was scored for the presence of significant hypercellularity, or evidence of fibrinoid necrosis or crescents.

The other kidney was immediately frozen in OCT embedding medium, and 3 contiguous 5-7 micron sections were stained with either directly FITC conjugated rabbit anti-mouse IgG, or a FITC conjugated polyclonal rabbit anti $Id^{LN}F1$ antiserum, or a FITC conjugated antibody against an $Id^{LN}F1$ positive monoclonal antibody.

3. Results.

A. Kidney analysis:

Histological analysis (as described above) of a series of control and treated kidneys from 6 month old male mice treated 5 times with either 30 μg/kg of TCDD, or with an equal volume/weight of olive oil revealed a proliferative glomerulonephritis involving significant numbers of glomeruli in most of the kidneys of treated, and some of the kidneys of control animals. Marked proliferation was seen in 7 out of 9 mice treated with five monthly doses of 30 μg/kg of TCDD. Less extensive proliferation was seen in one other treated and 2 out of 5 age matched oil treated controls. Four of the TCDD treated mice also showed fibrinoid necrosis or crescent formation reflecting a severe inflammatory glomerulonephritis. These latter lesions were not seen in any controls examined.

In some TCDD treated animals a persistent proteinurea was observed, and two animals in the above group became seriously edematous so that their weight was substantially increased. These abnormalities also, were not seen in the above controls.

In most cases where immunofluorescence analysis was possible, deposition of idiotype specific ($Id^{LN}F1$) immunoglobulin in the kidneys correlated positively with the histopathology, although there was an occasional (2 animals) disagreement in degree. Immunofluorescent analysis has thus far not been done in a completely blinded protocol. While deposition does not equal disease, the correlation between these two independent assays provides further support for the importance of the idiotype in nephritic pathogenesis in this model much as it has been confirmed in the female model.

B. Other histopathology.

Examination of H and E stained cross sections of the small intestine at the proximal end of the organ did not reveal a characteristic lesion in an initial blinded protocol. In some cases, TCDD treated animals appeared to have elevated numbers of mononuclear cells in cross sections of the villi, but this finding did not correlate with kidney pathology. Other abnormalities such as clumping or loss of villi did not correlate with treatment.

In some TCDD treated adult mice (both at 30 and 80 μg/kg), and in 3 out of 4 male perinatally exposed (80 μg/kg per dam) mice, large axillary or inguinal nodes were seen. Histopathology of the nodes suggested a "reactive" rather than a malignant phenotype. Immunophenotyping of the nodes revealed more than 60% of the cells were B-cells, and the remainder were T cells of both the CD4 and CD8 phenotype. Normal nodes in mice of this age and strain contain less than 40% B cells and 60% or more T-cells. Whether the B-cells in the enlarged nodes represent a monoclonal, oligoclonal, or polyclonal population has not yet been determined.

C. Immunological alterations.

We have not found a significant and consistent alteration in thymocyte phenotypes (CD3, CD4 and CD8) in treated vs. control mice as has been previously reported by us in single dose studies [5]. No statistically significant thymic atrophy was observed at the lower doses of TCDD. However, in both the spleen and liver of treated animals, we have observed an expansion of CD4 positive T-cells that lack expression of CD45RB, and express CD44. This phenotype has been demonstrated to be the phenotype of memory T-cells[13,14] in other mouse strains. Recent analysis of T-cell clones derived from the spleens of diseased female mice suggests this CD4+, CD44+, CD45 RB- phenotype may be the pathogenic T-cell phenotype. All clones thus far examined which can cause disease acceleration when injected into young female mice have this phenotype. It is also striking that this altered phenotype is observed in the liver compartment. Some research, including our own, has suggested that potentially autoreactive T-cells increase in the liver of animals treated with thymic atrophy inducing agents[6,15]. A similar expansion of the memory phenotype was observed in male perinatally exposed mice, at 18 weeks of age (15 weeks after exposure ceased).

In the intestinal epithelial lymphocyte compartment, we did not detect a definitive immunological alteration. However, in some of the mice that were manifesting acute pathology, we detected less than 1% CD4 positive cells, while age matched, oil treated controls had levels of 5-15%.

4. Discussion

Autoimmune disease is a complex phenomenon, with a substantial genetic component. It has long been recognized that estrogen and estrogen like compounds can promote such disease, but this probably requires other genetic predisposing factors[16]. Thus far no one has reported whether dioxin can promote autoimmune disease in an animal model, and the human evidence is anecdotal and insufficient. Although TCDD has been noted to induce hydronephrosis and other kidney damage during fetal and perinatal exposure[17,18] and some forms of kidney damage have been observed in adult mice[19,20] at high doses, the lesions observed here are not similar to those reported in mice or rats. The lesions are, however, identical to those found in female SNF1 mice which develop the lupus like nephritis.

By exploring whether TCDD can accelerate or amplify autoimmune disease in a genetic background that is well characterized both histologically, and immunologically, we are finding a strong suggestion that TCDD could, in the appropriate genetic context, promote autoimmunity. The fact that this is observed in a strain that is more resistant to TCDD effects should give us some pause. The equivalent levels in a sensitive mouse strain would range from 0.5 to 5 µg/kg, once a month for 3-4 months. It is possible that these effects could be seen at even lower or fewer doses, and such studies are currently underway. The fact that some of these abnormalities are also seen in perinatally exposed mice 4 months after exposure is also of considerable interest.

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5. Bibliography

1) Kerkvliet N.(1994): "Immunotoxicology of Dioxin and Related Chemicals" in Dioxins and Health, ed. A. Schechter , Plenum Press, NY. pp. 199-225.
2) Umbreit, T.H. and Gallo, M.A. (1988) Physiological implications of estrogen receptor modulation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Tox. Lett. 42, 5-14.
3) Silverstone A.E.,N.C. Fiore, J.A.Soults, L.M.Cunningham, and T.A.Gasiewicz (1992): 2,3,7,8-Tetrachlorodibenzo-p-dioxin effects on lymphocyte stem cells: Comparisons with corticosteroids and estrogens. Chemosphere **25**.103-106.
4) Silverstone A.E., D.E. Frazier DE, Jr, N.C. Fiore,J.A. Soults and T.A. Gasiewicz (1994): Dexamethasone, ß-Estradiol, and 2,3,7,8-Tetrachlorodibenzo-p-dioxin elicit thymic atrophy through Different Cellular Targets. Toxicology and Applied Pharmacology, **126**. 248-259.
5) Frazier D.E.Jr, A.E.Silverstone, T.A.Gasiewicz (1994): 2,3,7,8-Tetrachlorodibenzo-p-dioxin-Induced Thymic Atrophy and Lymphocyte Stem Cell Alterations by Mechanisms Independent of the Estrogen Receptor. Biochemical Pharmacology. **47**. 2039 - 2048.
6) Silverstone A.E., T.A.Gasiewicz, and D.E. Frazier Jr. (1994): Alternate Immune System Targets for TCDD: Lymphocyte Stem Cells and Extrathymic T-Cell Development. Exp. and Clin. Immunogenetics. **11**. 94-101.
7) Jennings A.M., G. Wild, J.D. Ward, and A.M.Ward (1988): Immunological abnormalities 17 years after accidental exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Brit. J. Ind. Med.**45**. 701-704.
8) Roegner R.H. and R.W.Ogershok(1991): Air Force Health Study: An epidemiologic investigation of health effects in air force personnel following exposure to herbicides. Vol. 7. *Serum dioxin analysis of 1987 examination results* Contract #F41689-85-/d-0010, Chapter 18.

9) Lu Y-C.and Y-C. Wu (1985): Clinical findings and immunological abnormalities in Yu-Cheng patients. Environ. Health Perspec.**59**. 17-19.
10) Hong R., Taylor, K., and Abonour, R. (1989) Immune abnormalities associated with chronic TCDD exposure. Chemosphere **18**. 313-320.
11) Knupp C.J., A.H.Uner, and J.Gavalchin (1992): The onset of nephritis in the (NZB X SWR)F_1 murine model for systemic lupus erythematosus correlates with an increase in the ratio of CD4 to CD8 T lymphocytes reactive for the nephritogenic idiotype ($Id^{LN}F_1$). Clin. Immunol. and Immunopathol.**65**.167-175.
12) Knupp C.J., A.H. Uner, C.Korthas, and J.Gavalchin, J.(1993): Characterization of $Id^{LN}F_1$-reactive CD4+ T cell clones from the (NZB X SWR)F_1 model for systemic lupus erythematosus. Clin. Immunol. Immunopathol. **68**.273-282.
13) Budd R.C., J-C. Cerottini, C. Horvath, C. Bron, T. Pedrazzini, R.C. Howe, and H.R. MacDonald (1987): Distinction of Virgin and Memory T Lymphocytes. Stable acquisition of the Pgp-1 glycoprotein concomittant with antigenic stimulation. J. Immunol. **138**. 3120-3129.
14) Beverley P.C., A. Daser, C.A. Michie, and D.L. Wallace (1992): Functional subsets of T cells defined by isoforms of CD45. Biochem. Soc. Transact. **20**. 184-187.
15) Okuyama R.,T. Abo, S. Seki, T. Ohteki, K. Sugiura, A. Kusumi, and K. Kumagai (1992): Estrogen administration activates extrathymic T cell differentiation in liver. J. Exp. Med.**175**. 661-669.
16) Ahmed S.A. and N. Talal (1988): Sex Steroids, sex steroid receptors, and autoimmune diseases in Steroid Receptors and Disease: Cancer, Autoimmune, Bone, and Circulatory Disorders. ed by P.J. Sheridan, K. Blum, and M.C. Trachtenberg, Marcel Dekker, NY pp. 290-316.
17) Moore J.A., B.N. Gupta, J.G. Zinkl, and J.G. Vos (1973): Postnatal Effects of Maternal Exposure to 2,3,7,8-Tetrachlorodibenzo-p-dioxin(TCDD). Env. Hlth. Perspec. **5**.81-85.
18) Abbott B.D., L.S. Birnbaum, and R.M. Pratt (1987): TCDD-Induced Hyperplasia of the Ureteral Epithelium Produces Hydronephrosis in Murine Fetuses. Teratol. **35**. 329-334
19) Kluwe W.M. and J.B. Hook (1980): Effects of environmental chemicals on kidney metabolism and function. Kidney Intl. **18**. 648-655.
20) McConnell E.E.(1980): Acute and chronic toxiciy, carcinogenesis, reproduction, teratogenesis and mutagenesis in animals. in Halogenated biphenyls, perphenyls, napthalenes, diobendioxins, and related products. R.D. Kimbrough, ed. Amsterdam, Elsevier. 109-150.