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CHAPTER 4

"THE FACT THAT TCDD IS ACUTELY TOXIC IS NOT IN DISPUTE, THE ISSUES ARE THE LEVELS OF EXPOSURE AND SPECIES SUSCEPTIBILITY."

ASSESSMENT OF THE ANIMAL TOXICOLOGIC DATA FOR TCDD

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The toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been well documented (1). The extreme toxicity of this substance has been noted by many authors, and some have compared its lethality to that of aflatoxin (1). This applies only in certain species, and there is considerable difference, for instance, in the LD50's in different animal species, as noted in Table 1. It has been shown that one animal, namely the beagle dog, is much more resistant than others to dioxin. The reason for this, some investigators suggest (19), is that dogs convert TCDD to its metabolites at a much higher rate, and the crude canine biliary metabolite is at least 100 times less potent in its toxic effect than TCDD itself. TCDD metabolites differ among individual animal species (19), and clearly this aspect of the problem requires further investigation, particularly as it relates to man. Considerable evidence suggests that humans are less dramatically affected by exposure to TCDD than other animal species (13).

Table 1. Acute LD_{50} in Selected Species of 2,3,7,8-Tetrachlorodibenzo-p-dioxin.

Species	Micrograms per Kilogram Body Weight
Guinea pig	0.6
Rat (S.D.)(Male)	22.0
Rat (S.D.)(Female)	45.0
Rhesus Monkey	Less than 70
Rabbit	115.0

The extreme toxicity of TCDD in rats and mice gives rise to very interesting and difficult problems with regard to long-term toxicity and carcinogenicity studies. It is usual when planning long-term studies of 18-24 months in rats and mice to establish a so-called maximum tolerated dose. The purpose of this is to achieve a study which will produce recognizable toxic effects, but will not cause death or severe damaging lesions over the period of the lengthy exposure to the drug or chemical. It is the purpose of this chapter to examine and discuss the difficulties surrounding the development of long-term studies and their interpretation, using a chemical which has such extreme lethality at very low doses.

CARCINOGENICITY

Several long-term studies in rats and mice have been performed using TCDD:

1. A seventy-eight week study in male Sprague-Dawley rats (ten males per group with TCDD administered in the diet)(30).

   In this study, nine dose groups were used, varying from an approximate intake of TCDD of 0.0003 micrograms/kilograms/week (mg/kg/wk). At the three upper dose levels of this study all the animals died within four weeks. At the remaining six dose levels the lowest (0.0003 mg/kg/wk) showed no evidence of tumors, while the other five dose levels, which varied from 0.001 mg/kg/wk to 2.0 mg/kg/wk, all showed evidence of a variety of tumors. At the upper levels hepatocellular carcinomas and thyroid carcinomas were described. This study is somewhat unsatisfactory from a number of points of view:

   a. Very small numbers of animals were used per group and only males were examined.

   b. At the upper dose levels, all the animals died.

c. The main problem was that in the controls the authors reported zero incidence of tumors. This seems highly unlikely in aging rats.

2. A two-year study in Sprague-Dawley rats, using 50 males and 50 females per group with the TCDD administered in the diet (4).

   In this study three dose levels were used: 0.001 mg/kg/day, 0.01 mg/kg/day, and 0.1 mg/kg/day. At the lowest dose there were no significant toxicity problems and no evidence of increased numbers of tumors compared with controls. At the mid dose there was evidence of increase in urinary porphyrins in females and an increase of hyperplastic nodules in the livers of females, but no actual carcinomas. At the 0.1 mg/kg/day level there was an increase in mortality with numerous toxic changes, including marked decrease of body weight gain and an increase of excretion of urinary porphyrins and delta aminolevulinic acid. In addition, there was an increase in serum alkaline phosphatase and SGPT. There were also a number of generalized defects, including lymphoid tissue hypoplasia. In this high dose group there was evidence of carcinogenesis including hepatocellular carcinomas in females and squamous cell carcinoma of the lung, hard palate and tongue of both sexes. In the controls of this study there was the usual scatter of expected tumors, both benign and malignant. One interesting feature was that certain tumors expected in an aging population of rats were decreased in number. There was also evidence of diminished numbers of pituitary changes and renal damage and infections.

3. Three studies have been reported by the National Toxicology Program (9).

a. A study performed in Osborne-Mendel rats by gavage in which carcinogenicity was reported with follicular cell carcinomas of the thyroid in males and carcinomas in the liver of females.

b. An oral study in B6C3F1 mice was considered carcinogenic with liver tumors in both sexes and thyroid tumors in females.

c. A dermal study performed in Swiss-Webster mice was considered carcinogenic in females but not in males. Integumentary fibrosarcomas were seen in females at a statistically significant level and, while these same tumors were increased in the male, there was no statistical significance when compared with controls.

The Kociba et al. (11) study is the one performed in a manner which can most readily be interpreted. It was preceded by a standard three-month dose range-finding study, and a reasonable number of tumors were found of the type one would expect in the control group. It seems interesting, therefore, to quote the commentary of the authors of this study, in which they say, "Doses sufficient to induce severe toxicity increase the incidence of some types of neoplasm in rats, while reducing the incidence of other types. No increase in neoplasms occurred in rats receiving sufficient TCDD during the two-year study to induce slight or no manifestations of toxicity."

While it is not possible fully to interpret these findings, they do identify some of the difficulties which are found in performing long-term carcinogenic studies in rats and mice. On the one hand there are those chemicals which have such a low degree of toxicity that relatively enormous doses have to be used before one can demonstrate significant tissue changes. A good example of this type of chemical is saccharine. On the other hand there are

those substances, of which TCDD is an excellent example, which are so extremely toxic that it is very difficult to achieve doses small enough to produce a satisfactory study without causing excessive manifestations of toxicity. Such compounds raise the question of whether the cell damage itself is related to the development of neoplasms.

It also raises the question of the relationship of overwhelming toxicity to tumor production. In the case of the dioxin study at the dose level of 0.1 mg/kg/day, where malignant tumors were found there were also very severe toxic changes, including depression of lymphoid tissue proliferation, abnormal excretions of substances such as porphyrins and marked decreases in body weight gain. It is very difficult at the present stage to interpret this in relation to potential human effect. Clearly the fact that tumors occur strikes a note of warning. When a new chemical is being developed for therapeutic use such a warning must be taken very seriously and risk versus benefit must be evaluated. When a contaminant of otherwise valuable substances,including herbicides, is under consideration, the problem is to decide whether to abandon all the chemicals concerned or to try to determine "a safe level" at which the contaminant, in this case dioxin, can be permitted. This in turn raises yet another interesting question. A number of observers have accepted the fact that TCDD is carcinogenic, but have stated that the findings do not show that TCDD is an initiator or a promoter (9,18.24). This further raises the question that, if TCDD could be shown to be a promoter, then the possibility of dose relationship to carcinogenicity becomes more likely, and also the relationship to intermittent exposure or removal of the hazards for certain individuals who have been subjected to exposure could represent safety measures.

TCDD has many interesting features related to its carcinogenic potential. Although it has been shown in the animal studies described above that it produces tumors, there is very inconclusive evidence that it is, in fact, a mutagen (5,6,10,26,29). There is also no good evidence to demonstrate that TCDD is metabolized to an electrophile or that it is capable of binding invivo covalently (23). The fact that TCDD cannot be proved to be a mutagen is considered by some to be evidence that it is not an initiator (18). Pitot and co-workers (18) have recently sought to show that TCDD acts as a tumor promoter. If such a finding is true, it might well be that this is the mechanism for the observations of tumorigenicity with this compound. Pitot et al. (18) used a so-called "two-stage" procedure. Partially hepatectomized rats were given a single dose of 10 mg/kg/bwt of diethylnitrosamine (DEN), which was a dose insufficient to produce liver carcinomas. This was followed by the administration of TCDD in doses of 0.14 and 1.4 mg/kg/bwt subcutaneously every two weeks for seven months. This procedure increased in a statistically significant manner the number of enzyme altered foci in the liver and also led to the production of hepatocellular carcinoma. The TCDD administered alone did not lead to carcinomas. Thus, in this two-stage model they believe that TCDD acts as a tumor promoter.

These findings are of interest, for the two-stage procedures for carcinoma determination, whether they are applied in the liver (17) or in the bladder (8,14), have been accepted by many as being significant. This opinion is based on the original findings of Berenblum and Shubik (2,3) in their classic experiments with the development of skin carcinoma, using an apparently similar model. It is interesting, therefore, to quote Shubik (27), who is once

again reminding experimentalists that this transposition of a concept cannot be justified without greater indepth studies of the many differences which are apparent between the two systems. He questions, for instance, whether in neoplasia "we are merely dealing with a general cellular mechanism that exists to cope with a variety of intracellular injuries?" He also comments on the question of tumor progression and its relationship, if any, to promotion. He further warns against drawing analogies between the older invivo studies with the very frequently used invitro systems. He points out that there is a possibility for oversimplification in such analogies which may lead to missing important aspects of the neoplastic process. TCDD certainly seems to be a chemical which gives rise to such questions. This is a substance which produces considerable cellular damage, which is associated with the development of malignant tumors of various types in certain animal species and possibly the reduction of the incidence of some naturally occurring tumors (11).

MECHANISMS OF TOXICITY

When considering the possible mechanism of toxicity for TCDD and related chemicals, the induction of microsomal monooxygenase activity and other enzymes expressed in similar circumstances has been most studied and is best understood at the present time.

This system metabolizes most foreign chemicals of lipophilic type which enter the body into a more readily excretable product. The enzyme complex is situated in the endoplasmic reticulum. It consists of flavoprotein NADPH-cytochrome P-450 reductase and a group of hemoproteins, which altogether are termed cytochromes P-450. These have been studied principally in the liver of rats and mice.

Seven distinct species of cytochrome P-450 have been identified in rat liver, four of which have been purified to homogeneity. They have distinct but often overlapping substrate specificities and are under independent genetic control (12). Two different chemicals have been used as prototype compounds for inducing different species of cytochrome P-450 and increasing different monooxygenase activities.

These chemicals are phenobarbital and 3-methylcholanthrene (MC) (4). The activity most frequently measured for MC response is arylhydrocarbon hydroxylase (AHH) activity. TCDD and related halogenated aromatic hydrocarbons produce an induction of MC pattern (24). It is therefore with the MC type response that we are interested. Induction of AHH activity by the various types of halogenated hydrocarbons does give a measure of their potency, and TCDD is the most potent and the most toxic of all these. It indicates, therefore, that there may be some relationship between the potency to induce AHH activity with the potential for toxicity. It has already been pointed out that MC is the prototype for inducing cytochrome P-450 and AHH activity. In a comparison of MC with TCDD for their capacities to induce hepatic AHH activity in rats, both compounds produce parallel dose-response curves and the same maximal enzyme induction, but TCDD is 30,000 times as potent as MC.

In continued work with the induction of heptic cytochrome P-450 and AHH activity with MC, it has been discovered that two types of mice exist, in terms of enzyme induction. One of these is responsive to MC and the other is not. The prototypes of this difference are the C57BL/6 strain, which is responsive, and the DBA/2 strain, which is non-responsive (15,20,28). In investigations of the background of this

phenomenon, the trait of responsiveness to aromatic hydrocarbon (that is, the induction of hepatic AHH activity by MC or other polycyclic aromatic hydrocarbons) is inherited as a simple dominant. The genetic locus that controls this trait has been designated Ah (for aromatic hydrocarbons) and the allele for responsiveness is denoted as Ahb (b for C57BL/6 strain mice) and the allele for non-responsiveness has been designated Ahd (d for DBA/2 type mice).

In view of this difference in responsiveness to MC and the fact that TCDD is so much more potent than MC, experiments have been performed with TCDD induction of AHH in both strains. The results, simply stated, are:

1. With the MC responsive strain good responses are achieved with TCDD.
2. With the non-responsive MC strain a moderate but satisfactory response is obtained with TCDD (21).

Further experiments have been performed to try to determine whether this is just a matter of potency or whether different receptors might be involved. The consensus of the results of these experiments is that the difference is one of potency (16,22). This is important because it indicates that a reasonably specific test can be used to evaluate potency of halogenated hydrocarbons of aromatic type and also that this can be related to their potential toxicity.

Although at first signt this appears to be somewhat theoretical information, it opens the door to very interesting possibilities of much more precise determination of toxic potential in the future, by applying specific experimental approaches which are related to the specific structure and activities of other groups of chemicals.

Thus, if there is an excellent correlation, as it appears that there is, between the toxic potency of halogenated hydrocarbons and their potency to induce AHH activity, then, the understanding of the mechanism of AHH induction might provide insight into our understanding of the mechanism of toxicity for these chemicals.

Further work has shown by a variety of experimental criteria that cytosol binding protein has the invitro properties expected of the receptor for the induction of cytochrome P-450 and AHH activity (9).

In addition, other work strongly suggests that the Ah locus (in mice) is the structural gene for cytosol receptor (24).

It would appear, therefore, that a pattern has been established for the mechanism by which substances such as MC and TCDD exert their enzyme stimulating effects and how they differ from other substances such as phenobarbital or pregnenolone 16-carbonitrile, another compound which produces a different pattern of cytochrome P-450 activity. The substance pregnenolone 16-carbonitrile is of some interest in this regard, as the pattern which it produces is similar to that which occurs with some steroid compounds.

Summarizing so far, it would seem that the similarities between structure and activity for toxicity with that for receptor binding suggest that the halogenated aromatic hydrocarbons including TCDD exert their toxicity through the cytosol receptor. However, many tissues other than liver, and, in addition, certain cell lines in culture, have receptors which respond to TCDD with the induction of AHH activity but show no evidence of toxic response. Thus, while the cytosol

receptor may be essential for toxicity, it is probably not the only mechanism to explain all the results seen. Of the various hypotheses to explain this problem, the most likely one to be correct seems to be that the induction of AHH activity may be viewed as a signal response but that it is not implicated directly in the mechanism of toxicity (24).

If toxicity of TCDD is mediated through the cytosol receptor then it should be expected that toxicity would segregate with the Ah locus, the gene which determines the cytosol receptor. In the case of thymus involution, teratogenesis and hepatic Porphyria (three toxic results of TCDD in mice) segregation with Ah locus has been determined in all three areas (24).

SUMMARY

Two independent lines of evidence suggest that toxicity of this group of hydrocarbons is mediated through their binding to the cytosol receptor: first, the correlation with the receptor binding and toxic potency, and second, the segregation with the Ah locus in three toxic responses produced by TCDD in mice. The results of a recent study (7) indicate that TCDD acts directly on epithelial target cells in the thymus. One consequence of this action appears to be the altered thymus-dependent maturation of T-lymphocyte precursors. These T-lymphocyte precursors are mediated through direct cell/cell contact between thymocytes and TE cells.

Poland and Knutson (24) have used this accumulation of information to develop a general model of toxicity using a so-called XB cell culture, which is a cloned mouse teratoma cell line (25) which produces dose-related characteristic

toxic responses with the group of chemicals under discussion. The details of their work are unnecessary in this brief discussion, but it does show how a sequence of specific investigations involving the detailed biochemistry and application of specifics relating a group of compounds to their toxic potential can be used to develop more precise methods for estimation of toxicity.

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