Published in final edited form as:

Toxicology. 2009 November 9; 265(1-2): 1–9. doi:10.1016/j.tox.2009.08.017.

Inflammatory and chloracne-like skin lesions in B6C3F1 mice exposed to 3,3',4,4'-tetrachloroazobenzene for 2 years

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Abstract

Exposure to dioxin and dioxin-like compounds (DLCs) has been connected to the induction of chloracne in humans and animals. 3,3',4,4'-Tetrachloroazobenzene (TCAB) is an environmental contaminant that induces chloracne in humans. TCAB has been studied only to a limited extent in laboratory animals. While performing a 2-year gavage study in B6C3F1 mice to evaluate the toxic and carcinogenic effects of TCAB, we also explored potential chloracnegenic properties. Groups of 50 male and 50 female B6C3F1 mice were exposed by gavage to TCAB at dose levels of 0, 3, 10 and 30 mg/kg for 5 days a week for 2 years. The animals developed treatment-related gross inflammatory skin lesions, which were characterized histologically by inflammation, fibrosis, hyperplasia, and ulcers. Additionally, many of the animals developed follicular dilatation and sebaceous-gland atrophy, consistent with chloracne-like lesions. This current 2-year study supports recently published papers showing susceptibility to chloracne in mouse strains other than hairless mice. The chloracne-like lesions were not clinically evident; therefore, our study highlights the need for careful examination of the skin in order to identify subtle lesions consistent with chloracne-like changes in rodents exposed to dioxin and DLCs. Since previous short term studies did not demonstrate any skin lesions, we suggest that reliable assessment of all safety issues involving dioxin and DLCs requires evaluation following chronic exposure. Such studies in animal models will help to elucidate the mechanisms of dioxin-related health hazards.

Keywords

TCAB; dioxin; chloracne; mice; inflammation

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Conflict of interest statement: The authors declare they have no competing financial interests.

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1. Introduction

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), commonly referred to as dioxin, is one of the most toxic compounds known, presenting both carcinogenic and teratogenic properties (Johnson, 1991; Kociba and Schwetz, 1982; Safe, 1990). Certain polychlorinated dibenzodioxins, polychlorinated dibenzofurans (PCDFs), and coplanar polychlorinated biphenyls (PCBs) are commonly referred to as dioxin-like compounds (DLCs) because they have the ability to bind to the aryl hydrocarbon receptor (AhR) and exhibit biologic actions similar to those of TCDD. DLCs induce developmental, endocrine, and immunological toxicity and multi-organ carcinogenicity in animals and/or humans (ATSDR, 1998, 2000; Bertazzi et al., 2001; Kociba et al., 1978; Steenland et al., 2001). In addition, DLCs induce chloracne, an acneiform skin eruption.

Chloracne, one of the most common occupational dermatoses, can be extremely refractory to treatment (Taylor, 1974) and last for long periods without additional exposure to chloracnogens (Tindall, 1985). Although the clinical features of chloracne are clearly defined, the cellular and molecular mechanisms of dioxin-induced chloracne remain unknown (Panteleyev and Bickers, 2006). This condition, always a symptom of systemic poisoning and not merely a cutaneous disorder (Pastor et al., 2002), is a well known side-effect in several dioxin-exposure accidents, such as those at Seveso, Yusho, and Yu-Cheng (Guo et al., 1999; Guo et al., 2004; Schecter et al., 2006; Urabe and Asahi, 1985). Recently, deliberate poisoning with TCDD caused Ukraine president Viktor Yushchenko to develop facial chloracne during his presidential campaign (Holt, 2005; Schecter et al., 2006; Sterling and Hanke, 2005).

The National Toxicology Program (NTP) recently conducted 2-year bioassays in female Sprague-Dawley rats to evaluate the chronic toxicity and carcinogenicity induced by dioxin, structurally-related PCDFs and PCBs and mixtures of these compounds, including a mixture of TCDD, 3,3',4,4',5-pentachlorobiphenyl (PCB126) and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) (NTP, 2006a, 2006b, 2006c, 2006d). In these studies, increases occurred in the incidences of neoplasms and nonneoplastic lesions in several organs, notably the liver, lung, and oral mucosa (Hailey et al., 2005; Jokinen et al., 2003; NTP, 2006a, 2006b, 2006c, 2006d; Nyska et al. 2004; Nyska et al., 2005; Tani et al., 2004; Walker et al., 2005; Walker et al., 2006; Yoshizawa et al., 2005a; Yoshizawa et al., 2005b). However, none of these rat studies revealed chloracne-like skin lesions, consistent with the fact that rat species are not sensitive to dioxin-induced chloracne (Greene et al., 2003).

The NTP also conducted a 2-year chronic gavage study using both rat and mice treated with 3,3',4,4'-tetrachloroazobenzene (TCAB), a DLC formed as a byproduct during the manufacture of 3,4-dichloroaniline or its herbicidal derivatives (Bunce et al., 1979; Hill et al., 1981; Poland et al., 1976; Sundstrom et al., 1978). Environmental contamination with TCAB occurs after chloranilide herbicides are degraded in soil by soil fungi (Bartha et al., 1968; Bartha and Pramer, 1969), and by photolysis and biolysis of 3,4-dichloroaniline (Mansour et al., 1975; Miller et al., 1980). Production of TCAB is estimated to be as high as 16,000 kg in the U.S. and may account for a significant amount of dioxin-like activity in the environment (Van Birgelen et al., 1999a). Occupational exposure to TCAB may occur during the manufacture as well as the application of herbicides containing TCAB as a contaminant. There is also the potential for human exposure via the consumption of food contaminated with TCAB.

In the *trans* configuration, TCAB can assume a planar conformation similar to that of TCDD and, like TCDD, can induce aryl hydrocarbon hydroxylase activity in mice and chick embryos (Poland et al., 1976). In several studies conducted in animals, exposure to TCAB caused typical dioxin-like effects, including body-weight loss, thymic atrophy, hepatotoxicity, anemia, developmental toxicity, and induction of cytochrome P450 1A1; an increase of porphyrins

occurred in chick embryo-liver cell cultures (Hsia et al., 1980; Hsia et al., 1981; Hsia et al., 1982; Hsia and Kreamer, 1985; McMillan et al., 1990; Mensink and Strik, 1982; Schrankel et al., 1982).

Three outbreaks of chloracne have occurred among workers following exposure to TCAB (Morse and Baker, 1977; Morse et al., 1979; Scarisbrick and Martin, 1981) in chemical plants manufacturing 3,4-dichloroaniline or its derivatives. Chloracne developed in 34 to 61 percent of the exposed workers. Although chloracne is the primary adverse effect reported in humans, only one animal study revealed TCAB-induced chloracne-like lesions following painting of the inner surface of the ears of female New Zealand White rabbits with TCAB for 5 days (Hill et al., 1981). The reason for this paucity of skin effects in published animal studies is that most were conducted with rats, a species resistant to dioxin-induced chloracne (Greene et al., 2003; NTP, 2006a, 2006b, 2006c, 2006d). In a subchronic rodent toxicity study conducted, in part, to set doses for the present 2-year study, TCAB was administered by gavage to B6C3F1 mice for 13 weeks. Although the treated mice showed typical dioxin-like effects in several organs, no effect was found in the skin (Van Birgelen et al., 1999a). However, chloracne-like lesions were observed in a 13-week toxicity study of tetrachloroazoxybenzene, a structural analog of TCAB (Van Birgelen et al., 1999b).

Despite its chloracnogenic properties in humans and close structural resemblance to TCDD, TCAB has only been studied to a limited extent in laboratory animals. Results in those few short-term studies did not show marked skin effects. Therefore, while performing the 2-year chronic gavage study in B6C3F1 mice for the evaluation of toxic and carcinogenic effects, we also explored the potential chloracnogenic properties of TCAB. Oral gavage was used for these studies allowing direct comparison of the data to the other NTP DLC toxicity studies. Here we report the skin findings in B6C3F1 male and female mice following exposure to TCAB for 2 years. The other significant neoplastic and nonneoplastic effects of TCAB are described in a NTP technical report (NTP, 2009).

2. Materials and Methods

2.1. Chemical

TCAB (CAS No. 14047-09-7) was obtained from AccuStandard, Inc. (New Haven, CT) in one lot. TCAB was identified by infrared and proton nuclear magnetic resonance spectroscopy, by gas chromatography (GC) coupled with mass spectrometry, and by melting point analysis. The purity of TCAB was determined by Karl Fischer titration to determine moisture content, elemental analysis for carbon, hydrogen, nitrogen and chlorine and GC with flame ionization detection, which indicated a purity 99.8% or greater. Purity of TCAB was analyzed several times during the study; no change was observed over the duration of the study. Dose formulations were prepared for administration by gavage by mixing TCAB in a corn-oil vehicle containing 1% USP-grade acetone; they were prepared fresh approximately once a month and were shown to be stable during that period of time. Pre- and post-administration analyses were performed using a GC assay with electron capture detection. All analyzed dose formulations were within 10% of the target concentrations.

2.2. Animals and housing

The studies were conducted in the AAALAC-accredited facility of Battelle-Columbus Laboratories (Columbus, OH). Animal use was in accordance with the United States Public Health Service policy on humane care and use of laboratory animals and the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). In addition, these studies were conducted in compliance with the Food and Drug Administration Good Laboratory Practice Regulations (Food and Drug Administration, 1987). Animals were male and female

B6C3F1 mice (Taconic Laboratory Animals and Services, Germantown, NY) approximately 4 weeks of age upon receipt; they underwent health screening during a quarantine period of about two weeks and were released for study when about 6 weeks old. They were randomly assigned to their respective experimental groups and permanently identified by tail tattoo. Male mice were housed individually, and female mice were housed 5 per cage; all were kept in solid-bottom polycarbonate cages (Lab Products, Inc., Seaford, DE) suspended on stainless steel racks. Filtered room air was supplied at the rate of at least 10 room-changes per hour. The mice were maintained at $72^{\circ} \pm 3^{\circ}\text{F}$, with a relative humidity of $50 \pm 15\%$ and a light/dark cycle of 12 hours each. Tap water and feed (irradiated NTP-2000 pelleted diet, Zeigler Bros., Inc., Gardeners, PA) were available *ad libitum*.

2.3. Experimental design

Groups of 50 male and 50 female mice were administered 0, 3, 10, or 30 mg TCAB/kg by gavage in a corn oil:acetone (99:1), 5 days per week for 105 weeks. The total dosing volume was 10 ml/kg body weight. The dose levels were selected based on the results from NTP 3-month toxicity studies (NTP, 1998; Van Birgelen et al., 1999a). All mice were observed twice daily for morbidity and once a month for formal clinical signs of toxicity. Moribund animals were sacrificed and necropsied. Health monitoring via sentinel animals showed no evidence of any significant rodent pathogens.

2.4. Pathology

Animals were euthanized by carbon dioxide asphyxiation. Complete necropsies were performed on all animals that died early or were killed at termination using standardized methodology. At necropsy, tissues, including masses and macroscopic abnormalities, were removed and fixed in 10% neutral buffered formalin. Skin samples were taken from clinically observed gross skin lesions and routinely from the dorsal skin of all animals. After fixation, tissues were trimmed, processed, embedded in paraffin, sectioned to a thickness of 5–6 μm , stained with hematoxylin and eosin (H&E) and examined microscopically.

The microscopic inflammatory skin lesions were graded using a semi-quantitative scale of 0–4, based on subjective evaluation of the number of inflammatory cells, where 0 = within normal limits; 1 = minimal alteration, barely exceeding normal variations; 2 = mild, easily seen but of negligible biologic impact; 3 = moderate, of large size and potential biologic impact; and 4 = marked, essentially maximum severity.

The follicular dilatation seen in the dorsal skin sections was graded on a four-point scale: 0 = no follicular dilatation; 1 = minimal dilatation with 2–3 follicles segmentally dilated to greater than double the normal diameter within a low magnification 4X field; 2 = mild with 4–5 follicles dilated as described; 3 = moderate with 6 or more follicles dilated as described.

The sebaceous gland atrophy seen in the dorsal skin sections was graded on a five-point scale: 0 = no sebaceous gland atrophy; 1 = minimal, total sebaceous gland area reduced by approximately 10%; 2 = mild, total sebaceous gland area reduced by up to approximately 33%; 3 = moderate, total sebaceous gland area reduced by up to approximately 50%; 4 = marked, total sebaceous gland area reduced by more than approximately 50%.

Additional details regarding the pathology data generation, quality assurance review and NTP pathology working group are available elsewhere (NTP, 2009). Details for the review procedures have been described (Boorman et al., 1985).

2.5. Statistical analysis

The incidences of microscopic lesions were evaluated statistically by the poly-3 test (Bailer and Portier, 1988; Portier and Bailer, 1989), which makes adjustments for survival differences among groups. Severity scores were compared among dose groups using Kruskal-Wallis analysis of variance for an overall comparison, and Mann-Whitney tests to compare each dose group to the control group (Hollander and Wolfe, 1973). Incidences of gross skin lesions were evaluated using the Cochran-Armitage trend test and Fisher's exact test to compare each dose group to the control group.

3. Results

3.1. Survival

Survival of male mice exposed to 10 and 30 mg/kg/day TCAB and female mice exposed to 30 mg/kg/day TCAB was significantly less than that of the vehicle controls (NTP, 2009). All males exposed to 30 mg/kg/day TCAB died before the end of the study. The survival rates in the 0, 3, 10, and 30 mg/kg/day male dose groups were 72, 62, 11, and 0 percent, respectively. The last surviving male mouse in the 30 mg/kg/day dose group died during week 76. Survival rates in the 0, 3, 10, and 30 mg/kg/day female dose groups were 71, 60, 65, and 39 percent, respectively.

3.2. Inflammatory skin lesions

During the 2-year chronic gavage study of TCAB, the only treatment-related clinically observed effect was the appearance of ulcers/abscesses. Grossly, these skin lesions appeared to be areas of discoloration, ulceration, or thickening, and were located predominantly in the head and neck region (Table 1). The incidence of gross skin lesions was significantly increased in the high dose females (Table 1). In the male mice, the incidences of gross lesions were significantly increased in the mid-dose animals but occurred at lower incidences in the high dose animals. The low incidence of skin lesions in the high dose male mice was attributed to the large number of early deaths in this group.

Histologically, the lesions were characterized by a combination of chronic active inflammation, dermal fibrosis and epidermal hyperplasia and ulceration (Fig. 1, Tables 2 and 3). In female mice, the incidence of these lesions was dose related and were significantly increased in the 30 mg/kg dose group relative to the vehicle controls. In males, the incidences of these lesions were significantly increased in all dose groups except for dermal fibrosis in the high dose group. There was no significant difference in the severity of the inflammatory skin lesions between the control group and the dosed groups (Tables 2 and 3). Lesions in the head and neck region were frequently accompanied by persistent scratching by the animal, which often caused exacerbation of the lesions, ultimately resulting in local ulcerations and worsening of the skin condition.

The time when the gross ulcers/abscesses first appeared was dose related. In male mice, lesions appeared first in the high and mid-dose groups, then in the low dose group and finally in the control group (Fig. 2A). In the female mice, lesions appeared first in the high dose group and last in the low dose group (Fig. 2B).

3.3. Chloracne-like changes

Dorsal skin samples are taken routinely from all animals in NTP studies for histological evaluation. No clinical or macroscopic abnormality was reported at this site. However, histopathologic examination of these samples revealed treatment-related changes in both sexes. The most obvious morphologic change was cystic dilatation of the infundibular segment of hair follicle (Fig. 3). The lumens of these dilated follicles contained variable quantities of

delicate, desquamated keratin particles, and the lining follicular keratinocytes were generally more flattened squamous cells compared to the more normally present low cuboidal keratinocytes. The incidences of follicular dilatation were significantly increased in both males and females exposed to 10 and 30 mg/kg/day TCAB (Table 4). Severity scores, however, did not differ across dose groups for males or females. This dilatation was not accompanied by the presence of inflammatory cells.

The associated sebaceous glands were either normal, atrophic due to reduced numbers of cells, or were completely absent (Fig. 3). The incidences of sebaceous gland atrophy were significantly increased in males exposed to 10 and 30 mg/kg/day TCAB and in females exposed to 30 mg/kg/day TCAB. Severity scores did not differ across dose groups for males or females (Table 5). No statistical correlation was found between the presence of follicular dilation and the presence of sebaceous gland atrophy.

Notably, the highest incidence of chloracne-like lesions, i.e. sebaceous gland atrophy and follicular dilatation, was found in the high dose male mice. This is in contrast to the low incidence of inflammatory lesions seen in this group attributed to the high mortality incidence. In addition, differences in chloracne-like lesion incidence between male and female mice were less pronounced relative to the difference in inflammatory lesions between the genders.

4. Discussion

We have shown that administration of TCAB for two years induced two distinct kinds of lesions in the skin of B6C3F1 mice. Skin samples were not taken from grossly apparent skin lesions but rather from apparently normal dorsal skin following standard NTP procedures. In the 13-week gavage study of TCAB, neither macroscopic nor microscopic skin lesions were observed (Van Birgelen et al., 1999a) indicating that long-term studies may be needed to detect TCAB-induced skin pathology. Incidences of nonneoplastic skin lesions characteristic of chloracne, including follicular dilatation and sebaceous gland atrophy, were significantly increased in male and female mice. Atrophy of sebaceous glands likely occurred secondary to follicular occlusion (Yamamoto and Tokura, 2003). The morphological characteristics of these lesions were consistent with chloracne-like lesions observed in humans and animals following exposure to TCDD and DLCs. Human chloracne is primarily characterized histologically by an acne-like eruption of comedones (Yamamoto and Tokura, 2003). The presence of non-inflammatory infundibular cysts accompanied by sebaceous gland atrophy is consistent with long-standing lesions, and their presence is considered pathognomonic for advanced chloracne (Panteleyev and Bickers, 2006). The absence of inflammatory cells reflects another feature typical of chloracne (Coenraads et al., 1994).

Hill et al. (1981) noted hyperproliferation of the epithelium as a criterion for chloracne lesions in animals. Our study did not show hyperproliferation, but rather thinning of the epithelium. This is consistent with the timeline of chloracne lesion formation which starts with hyperproliferation of keratinocytes, but later thinning of the epidermis is apparent (Hambrick, 1957). The thin follicular epithelium in the dilated follicles may be the result of a specific effect of TCAB on the squamous terminal differentiation of the follicular epithelium (Yamamoto and Tokura, 2003), and is not considered to be secondary to the pressure of the accumulated keratin material observed within the lumen of the hair follicles.

Recently, a comprehensive model was formulated to explain the morphological changes seen in chloracne (Panteleyev and Bickers, 2006). According to this model, chloracne-induced transformation of the pilosebaceous unit is driven by activation and accelerated exit of cells from the stem cell compartment coupled with a shift from a pilosebaceous differentiation pattern to a keratinized squamous phenotype resulting in keratinized comedones. Since

chloracnogens affect stem cell renewal, chloracne becomes a persistent process that remains years after chemical exposure (Scerri et al., 1995).

In animals, the effects of dioxins and DLCs on skin vary among species. Chloracne-like lesions that develop after exposure to dioxin and DLCs have been observed in rabbits, monkeys, rhino mice, and hairless mice (Hill et al., 1981). The hairless mouse (*hr/hr* mutant) and other mice with skin mutations were considered to be the sole mouse strains sensitive to dioxin-induced chloracne lesions (Poland et al., 1984). Hairlessness, or the expression of the baldness phenotype, is not the sole reason for this susceptibility to TCDD because the ears of mice, which are normally hairless, do not exhibit histologic changes following TCDD administration (Knutson and Poland, 1982). Researchers have postulated that an interaction between the normal *hr* protein and the AhR provides protection against dioxin-induced toxicity in the skin and that the lack of this interaction in hairless mice results in increased sensitivity to dioxin-induced chloracne (Knutson and Poland, 1982; Cachon-Gonzalez et al., 1994; Fernandez-Salguero et al., 1995).

Typically, chloracne-like skin lesions have not been observed in haired mice, rats, or guinea pigs (Allen et al., 1977; Hambrick, 1957; Hebert et al., 1990; Horton and Yearly, 1985; McConnell et al., 1979; McConnell and Moore, 1979; van den Berg et al., 1988; Vos and Beems, 1971; Vos et al., 1982). In the current study, we demonstrate that B6C3F1 mice are susceptible to the development of chloracne-like lesions. A 13-week toxicity study of tetrachloroazoxybenzene, a structural analog of TCAB, also demonstrated chloracne-like lesions in gavaged B6C3F1 mice (Van Birgelen et al., 1999b). In addition, TCDD induces involution of sebaceous glands in haired mice, although without other chloracne characteristics (Puhvel and Sakamoto, 1988). Similar to prior NTP studies of DLCs (NTP, 2006a, 2006b, 2006c, 2006d), male and female Sprague-Dawley rats administered TCAB for two years did not develop chloracne-like or inflammatory skin lesions, although their dorsal skin was also routinely examined (NTP, 2009). No good explanation exists for these interspecies differences (Panteleyev and Bickers, 2006).

Most of the toxic biologic effects of TCDD are mediated by its specific binding to the AhR, a ligand-activated transcription factor in the cytosol (Panteleyev et al., 1997). Binding of dioxin to AhR leads to nuclear translocation of the protein complex, followed by binding to dioxin-responsive elements present in the genome (Hankinson, 1995). In this manner, the AhR transcriptionally up-regulates a battery of genes involved in xenobiotic metabolism and leads to the elimination of these xenobiotics by decreasing their half-lives (Ray and Swanson, 2004). A relative potency value for compounds that act through the AhR can be estimated by comparing binding affinity to the AhR compared to TCDD. Using this method the relative potency value for TCAB was found to be 0.2 (Poland et al., 1976a). Although the fundamental AhR-dependent mechanism by which TCDD induces gene expression is well understood, the process by which it causes skin lesions such as chloracne requires elucidation (Loertscher et al., 2001).

In addition to chloracne-like lesions, mice in this 2-year study also developed inflammatory lesions that were dose-dependent and regarded as treatment-related. Sporadic cases of inflammatory lesions, characterized by chronic active inflammation, dermal fibrosis, epidermal hyperplasia, and ulceration, are observed occasionally in vehicle control male mice in NTP studies, the cause of which is unknown. These lesions are not associated with the chloracne lesions that develop after dioxin exposure (Coenraads et al., 1994; Yamamoto and Tokura, 2003). Similar to the chloracne-like lesions, inflammatory skin lesions were observed only after long-term administration of TCAB (after week 20).

Polycyclic aromatic hydrocarbons (PAHs) are potent inducers of AhR activity (Hankinson, 1995) and elicit inflammatory skin responses, such as dermatitis or contact hypersensitivity (Wu et al., 2003; Yamamoto and Tokura, 2003). These chemicals may exert their inflammatory effects either as primary irritants or by allergic mechanisms (Anderson et al., 1995; Davila et al., 1995; Yamamoto and Tokura, 2003), which might be enhanced by reactive oxygen species generated by oxygenated PAHs (Bonvallot et al., 2001; Kepley et al., 2003). Polychlorinated biphenyls also induced inflammatory skin lesions in mice after 3 to 4 months of continuous oral administration (Nishizumi, 1970). Using transgenic mice, investigators recently demonstrated that constitutive activation of the AhR pathway alone can induce severe skin lesions with itching that mimic atopic dermatitis and contact hypersensitivity (Tauchi et al., 2005).

In conclusion, the gavage exposure of B6C3F1 mice to TCAB for 2 years induced two kinds of skin lesions – inflammatory lesions, consistent with dermatitis, and hair follicle dilatation accompanied by sebaceous gland atrophy, consistent with characteristics of chloracne-like lesions. Our study demonstrates that mice other than hairless are susceptible to chloracnogens. Since a 13-week study using the same methods employed in this study did not demonstrate any skin lesions, we suggest that reliable assessment of all safety issues involving dioxin and DLCs requires evaluation following chronic exposure. In addition, since the chloracne-like lesions were observed only microscopically in dorsal skin sections, and were not grossly evident, we suggest routinely collecting and evaluating skin samples from clinically normal skin.

Acknowledgments

The authors gratefully acknowledge Ms. JoAnne Johnson, Dr. Robert Sills, and Dr. Nigel Walker from the NTP/ NIEHS for their critical review of the manuscript. This research was supported [in part] by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences under Research Project Number 1 Z01 ES045004-11 BB.

Abbreviations

AhR	aryl hydrocarbon receptor
DLC	dioxin-like compound
GC	gas chromatography
NTP	National Toxicology Program
PAH	polycyclic aromatic hydrocarbon
PCBs	polychlorinated biphenyls
PCB126	3,3',4,4',5-pentachlorobiphenyl
PCDFs	polychlorinated dibenzofurans

PeCDF

2,3,4,7,8-pentachlorodibenzofuran

TCAB

3,3',4,4'-tetrachloroazobenzene

TCDD2,3,7,8-tetrachlorodibenzo-*p*-dioxin**References**

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Fig. 1. Inflammatory skin lesions. (A) Control mouse microscopy. Normal aspect of skin from a control animal. (B) Treated mouse microscopy. Histological section of skin from a female B6C3F1 mouse treated with 30 mg/kg of 3,3',4,4'-tetrachloroazobenzene in the 2 year study. The mouse exhibited macroscopic ulceration on the torso, which histologically showed chronic active inflammation, epithelial hyperplasia (blue arrow), ulceration (black arrows), and dermal fibrosis (star). H&E.

Fig. 2. Appearance timeline of ulcers/abscesses. Number of animals with grossly observed ulcers/abscesses over the study period.

Fig. 3. Chloracne-like skin lesions. (A, C, E) Control mouse dorsal skin. Histological section from a control male B6C3F1 mouse in the 2-year study with 3,3',4,4'-tetrachloroazobenzene. Hair follicles exhibit normality; adjacent sebaceous glands can be seen. H&E. (A) Black arrows indicate hair follicles. (C) Black arrows indicate hair follicles. Red arrows indicate sebaceous glands. (E) Black arrows indicate hair follicles. Red arrows indicate sebaceous glands. (B, D, F) Treated mouse dorsal skin. Histological section from male B6C3F1 mouse treated for 2 years with 30 mg/kg 3,3',4,4'-tetrachloroazobenzene. Note dilation of hair follicles, flattening of lining epithelium and atrophy of adjacent sebaceous glands. No infiltration of inflammatory cells can be discerned. This animal was assigned a moderate degree of hair follicle dilation

(i.e., 6 or more follicles segmentally dilated to greater than double the normal diameter within a low magnification field). H&E. (B) Black arrows indicate dilated hair follicles. (D) Black arrows indicate dilated hair follicles. Red arrows indicate atrophic sebaceous glands. (F) Black arrows indicate dilated hair follicles. Red arrows indicate atrophic sebaceous glands.

Table 1
Number of B6C3F1 mice with grossly observed ulcers/abscesses with body part distribution of the lesions by sex and dose group.

Dose group	Males						Females					
	0 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg	50 mg/kg	50 mg/kg	0 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg	50 mg/kg	50 mg/kg
Number examined	5	8	17	49	50	50	5	8	17	49	50	50
Head	3	4	2	0	0	0	1	2	4	12	12	12
Torso/Dorsal	1	1	0	0	0	0	1	0	1	0	1	1
Torso/Ventral	0	1	1	0	0	0	0	0	0	2	2	2
Torso/Lateral	0	1	1	0	0	0	0	0	0	2	1	1
Appendage	0	1	0	0	0	0	0	1	1	1	1	1
Total (Number of animals)	6	12	17	57	57	57	6	13	17	66	66	66

 $p < 0.01$

Table 2

Incidence of microscopic inflammatory skin lesions observed in male B6C3F1 mice by dose group.

Dose group	0 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Number of animals surviving to terminal sacrifice	35	31	5	0
Skin (Number of animals examined)	50	50	49	50
Inflammation, Chronic Active^a	3 (2.7) ^b	13 ** (3.3)	12 ** (3.3)	5 * (3.4)
Mild	1	1	0	0
Moderate	2	7	8	3
Marked	0	5	4	2
Dermis				
Fibrosis	3 (3.0)	12 ** (3.0)	12 ** (3.1)	4 (3.5)
Mild	1	3	4	0
Moderate	1	6	3	2
Marked	1	3	5	2
Epidermis				
Hyperplasia	3 (3.0)	13 ** (2.4)	12 ** (3.3)	6 * (2.8)
Minimal	0	2	0	0
Mild	0	4	0	2
Moderate	3	7	9	3
Marked	0	0	3	1
Ulcer	3 (4.0)	13 ** (4.0)	11 ** (4.0)	6 * (3.8)
Moderate	0	0	0	1
Marked	3	13	11	5

^a Number of animals with lesion.

^b Average severity grade of lesions in affected animals. Grading of lesions is described in the Materials and Methods section.

* $p < 0.05$;

** $p < 0.01$.

Table 3

Incidence of microscopic inflammatory skin lesions observed in female B6C3F1 mice by dose group.

Dose group	0 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Number of animals surviving to terminal sacrifice	35	30	32	20
Skin (Number of animals examined)	49	50	50	50
Inflammation, Chronic Active ^a	0	2 (3.0) ^b	6* (3.2)	11** (3.6)
Mild	0	1	2	0
Moderate	0	0	1	4
Marked	0	1	3	7
Dermis				
Fibrosis	0	1 (4.0)	4 (3.8)	11** (3.3)
Moderate	0	0	1	8
Marked	0	1	3	3
Epidermis				
Hyperplasia	1 (2.0)	2 (2.5)	4 (3.8)	11** (3.2)
Mild	1	1	0	1
Moderate	0	1	1	7
Marked	0	0	3	3
Ulcer	0	1 (4.0)	4 (4.0)	11** (4.0)
Marked	0	1	4	11

^a Number of animals with lesion.^b Average severity grade of lesions in affected animals. Grading of lesions is described in the Materials and Methods section.* $p < 0.05$;** $p < 0.01$.

Table 4

Incidence and severity grading of follicular dilatation in male and female B6C3F1 mice treated with TCAB.

Males				
Dose group	0 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Number of animals examined	50	50	49	50
Follicular dilatation^a	7 (15.1%) ^b	10 (23.2%)	13 [*] (38.6%)	28 ^{**} (87.1%)
Mean severity score^c	1.3	1.2	1.2	1.4
Females				
Dose group	0 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Number of animals examined	49	50	50	50
Follicular dilatation	2 (4.7%)	0 (0%)	11 ^{**} (25.0%)	23 ^{**} (54.4%)
Mean severity score	1.0	--	1.3	1.4

^a Number of animals with lesion.^b Overall rate (Number of animals with follicular dilatation per number of animals examined at necropsy adjusted for survival).^c Average severity grade of lesions in affected animals (see Materials and Methods section for details).^{*} $p < 0.05$;^{**} $p < 0.01$.

Table 5

Incidence and severity grading of sebaceous gland atrophy in male and female B6C3F1 mice treated with TCAB.

Males				
Dose group	0 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Number of animals examined	50	50	49	50
Sebaceous gland atrophy^a	13 (27.3%) ^b	10 (22.6%)	20 ^{**} (54.7%)	29 ^{**} (87.2%)
Mean severity score^c	2.8	2.1	2.6	2.2
Females				
Dose group	0 mg/kg	3 mg/kg	10 mg/kg	30 mg/kg
Number of animals examined	49	50	50	50
Sebaceous gland atrophy	6 (13.7%)	10 (21.5%)	11 (24.2%)	15 [*] (36.6%)
Mean severity score	2.2	2.7	2.9	3.0

^a Number of animals with lesion.^b Overall rate (Number of animals with sebaceous gland atrophy per number of animals examined at necropsy adjusted for survival).^c Average severity grade of lesions in affected animals (see Materials and Methods section for details).^{*} $p < 0.05$;^{**} $p < 0.01$.