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COMPARISON OF THE IMMUNOSUPPRESSIVE EFFECTS IN MICE OF 2,3,7,8-TETRA-  
CHLORODIBENZO-P-DIOXIN (TCDD) AND 2,3,7,8-TETRACHLORODIBENZOFURAN (TCDF)

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Proceedings of the Symp. on Chlorinated Dioxins and  
Dibenzofurans in the total environment.

L.H. Keith et al., Eds., Ann Arbor Science Publishers  
Inc., USA

Submitted, October, 1982

ABSTRACT

The immunosuppressive effect of TCDF on humoral antibody production was investigated in C57Bl/6 mice after a single injection. Maximum effect was obtained 7 days after TCDF treatment, the suppression being totally reversed by day 42. Dose-response analysis performed on day 7 showed that doses  $> 10\text{--}20\text{ }\mu\text{g/kg}$  significantly reduced the immune response. Moreover it was estimated that doses of TCDF 30 times higher than TCDD were equiactive on day 7. Recovery was faster than with TCDD, which was still highly depressive on day 42.

Studies in C57Bl/6 and DBA/2 mice, which differ in their expression of the TCDD specific cytosol receptor, showed that C57Bl/6 mice, which have the higher receptor level and/or affinity, were more suppressed than DBA/2 mice. These results could support the hypothesis that TCDF acts in vivo through the same receptor as TCDD.

## INTRODUCTION

Polychlorinated dibenzodioxins and dibenzofurans have been found to be persistent environmental contaminants. The 2,3,7,8-tetrachloro-isomers of both classes of compounds are highly toxic in laboratory animals (1,2). TCDD can be formed during the manufacture of 2,4,5-trichlorophenol and may thus occur as a contaminant of herbicides. Accidental poisoning of animals and humans has occurred over the years (3,4), as a result of the production and use of chlorophenol and chlorobenzene compounds (5). TCDF is present in polychlorinated dibenzofuran mixtures detected in treated wood and polychlorinated biphenyls (PCBs), including PCBs contaminating the rice oil responsible for the human intoxication known as Yusho sickness (6). TCDD and TCDF have also been found in fly ash and flue gases from municipal and industrial incinerators (7).

TCDF is closely related to TCDD in structure and acute toxicity (8).  $LD_{50}$  in the animal species investigated ranged from  $< 10 \mu\text{g/kg}$  for the guinea pig to  $> 6000 \mu\text{g/kg}$  for C57BL/6 mice (1). As reported for TCDD, signs of toxicity differ in different species, hepatic lesions being observed in rats and mice, but not in guinea pigs or monkeys (1). Progressive weight loss and thymic atrophy are common findings. It is believed that TCDF is as toxic as TCDD at doses 15-30 times those of TCDD (1).

The immunosuppressive effects of TCDD have been extensively studied in animals and it is a potent inhibitor of humoral and cell-mediated responses (9,10) even depressing pluripotent bone marrow stem cells if given during the perinatal period (11). The very limited data available on TCDF's immunosuppressive effects (12) suggest that they are similar to those of TCDD.

We have previously shown (9) that in adult mice low doses of TCDD depress humoral more than cellular responses and that strains of mice (DBA/2 and AKR), more resistant to TCDD induction of hepatic aryl hydrocarbon hydroxylase (AHH) enzymes, are also more resistant to the immuno-suppressive effects than very sensitive strains (C57BL/6 and C3H/He) (13,14). The AHH enzyme system plays a central role in the

biotransformation of aromatic xenobiotics, potentially carcinogenic metabolites being produced and/or degraded by this complex of enzymes (15,16).

In this study we compare the immunosuppressive activity of TCDD and TCDF in C57Bl/6 mice after single exposure, evaluating humoral antibody production. TCDF is the most powerful in vitro inhibitor of TCDD binding to the specific cytosolic receptor, responsible for AHH enzyme induction, and is thus believed to act through a similar mechanism of action. For this reason TCDF is studied here in C57Bl/6 and DBA/2 mice which differ in their level and/or affinity of TCDD specific receptor and are differently susceptible to TCDD's immunosuppressive effects (14,17).

#### MATERIALS AND METHODS

Mice : C57Bl/6 and DBA/2 mice, hereafter called respectively B6 and D2, were obtained from Charles River, Calco, Italy. All animals were used at 10-12 weeks of age.

Chemicals: TCDD obtained from Kor Isotopes, Cambridge, MA was given as a single i.p. or oral (p.o.) injection in a volume of 0.1 ml/10 g body weight of an acetone:corn oil solution (1:6 v/v). TCDF was a kind gift from Dr. C. Rappe, University of Umea, Sweden. It was dissolved and given as described for TCDD.

Response to sheep red blood cells: Mice (7-8 per group) were injected i.p. with  $4 \times 10^8$  sheep red blood cells (SRBC) 7, 21 or 42 days after drug treatment, and spleen hemolytic plaque-forming cells (PFC) were counted 5 days later (9).

Peritoneal cells: Peritoneal exudate cells (PEC) were obtained by washing the peritoneal cavity with 5 ml of PBS (Phosphate Buffered Saline). Differential counts were made with Turk's solution (18).

Statistical analysis: Results are presented as means  $\pm$  s.e. Statistical significance was analysed by Student's "t" test, or by Dunnett's test when more than two experimental groups were compared.

## R E S U L T S

Body and thymus weights and spleen and peritoneal cell numbers were evaluated as signs of general toxicity. Results are shown in Table 1. No effects on body weight were observed at any of the doses tested, whereas doses higher than 180  $\mu\text{g/kg}$  i.p. or p.o. significantly reduced thymus weights. This dose was also toxic for the spleen as shown by the lower number of splenocytes recovered after TCDF treatment. No significant modifications were seen in the number of total peritoneal cells and macrophages. Peritoneal cell and macrophage counts after i.p. treatment were higher than those from p.o. treated mice; it must be noted, however, that TCDF was given in an acetone:oil solution that probably has some local irritant effect.

Immunosuppressive effect was then investigated giving the toxic agent 7 days before antigenic stimulation. A wide range of doses was tested, from 5 to 900  $\mu\text{g/kg}$ . Fig. 1 reports the dose-response curve of TCDF inhibition of antibody production and the results with TCDD for comparison. Humoral response was similarly depressed by both compounds, the major difference being in the effective doses: about 30 times more TCDF than TCDD, in terms of  $\mu\text{g/kg}$ , were needed to depress the immune response to the same level.

Since the i.p. route is an experimentally easy, but not realistic route of contamination, TCDF was also given orally and antibody production was evaluated. Results, in Table 2, clearly show that the same degree of inhibition could be obtained with both treatments, in good agreement with previous reports for TCDD (9).

The time-course of immunosuppression was investigated next. Doses of

TCDD (6 µg/kg) and TCDF (180 µg/kg) found to be equiactive on day 7 were used. Results, reported in Fig. 2, show that 42 days after TCDD exposure the inhibition was still present and was as strong as that observed on day 7. In contrast the depression induced by TCDF had completely disappeared by day 42.

In B6 mice the whole body half-life of TCDF is 2 days and metabolites were found in urine and feces (19), while TCDD is not metabolized to an appreciable extent in the same animals, with a half-life of 17 days (20).

Because of the importance of the AHH enzyme system and the differences in TCDD immunosuppression in B6 and D2 mice which differ in the level and/or affinity of the specific receptor responsible for AHH induction by TCDD, TCDF was also investigated in these two mouse strains. Table 3 shows the body and thymus weights. Thymus weight was reduced only in B6 mice. When humoral response was investigated, 85% inhibition of antibody production was observed in B6 mice after treatment with both compounds, while only 40% inhibition was induced in D2 mice (Fig. 3). This difference between the two strains was confirmed by the number of PFC per million splenocytes (Fig. 3, left part).

## DISCUSSION

Results reported here show that TCDF has a definite immunosuppressive effect on humoral response in B6 mice. Similar degrees of immunosuppression can be obtained with TCDF doses 30 times higher than those of TCDD. However, because the LD50 for TCDF is not yet well defined (1), it is difficult to compare equisuppressive doses of TCDF and TCDD and fractions of LD50. Because the TCDF is > 6000 µg/kg and that of TCDD is 115-132 µg/kg (20,21), one can calculate that TCDF (180 µg/kg) is equiactive with TCDD (6 µg/kg) at a dose less than 1/30 of the LD50, while that of TCDD is 1/20. It might be concluded that TCDF in B6 mice should be more suppressive than TCDD, at least until definitive data on TCDF LD50 is available.

However when we looked at the time course of the immunosuppressive effect,



marked differences were observed between the two toxic agents. TCDD immunosuppression did not change from day 7 to day 42, whereas by day 42 TCDF suppression had completely disappeared. The two agents reportedly have very different half-lives (2 days for TCDF and 17 days for TCDD); in addition TCDF is metabolized and thus excreted faster, while no relevant metabolism of TCDD was observed in B6 mice (19,20).

Since in studies in the guinea pig the examination of immunological parameters stopped 7 days after the last dose (12), it is difficult to say whether the time course of TCDD and TCDF effects are similar or not in species other than mice.

Results in B6 and D2 mice, showing a good correlation between TCDD and TCDF effects may support the hypothesis that the TCDD receptor plays a role, not only in vitro (22), but also in vivo for the biological activity of TCDF (23). This receptor was shown to be present not only in the liver, but in other organs too (24), the highest concentration reportedly being in the thymus (24), the organ most affected by TCDD in all the species investigated. In line with this view are the data of Poland and coworkers (23) showing that the TCDD binding in vitro to thymic cytosol from D2 mice is far less than in B6. With the data on TCDF half-life in B6 and D2 mice, it has been reported (19) that similar levels of radioactivity were found in thymus and spleen after administration of labeled TCDF. This reasonably excludes that the differences observed in the two mouse strains were simply due to different levels of TCDF in thymus and spleen.

If TCDF interacts in some way with the TCDD receptor, inducing similar toxic effects, its binding should be shorter-lasting and/or weaker, as suggested by the half-life and by the time course of antibody production inhibition.

#### ACKNOWLEDGEMENTS

The generous contribution of the Gustav and Louise Pfeiffer Research Foundation, Los Angeles, Calif., USA, is gratefully acknowledged.

LEGENDS TO FIGURES

Fig. 1 : Dose-response effect on humoral antibody production in B6 mice of TCDD and TCDF given i.p. 7 days before the antigen ( $4 \times 10^8$  SRBC).

The test was performed 5 days after antigenic stimulation.

Fig. 2 : Time-course of the immunosuppressive effect of TCDD and TCDF given i.p. at days indicated. Immunization and test as in fig. 1.

Fig. 3 : Effect of TCDD and TCDF on humoral antibody production in B6 and D2 mice. Treatment, immunization and test as in fig. 1.

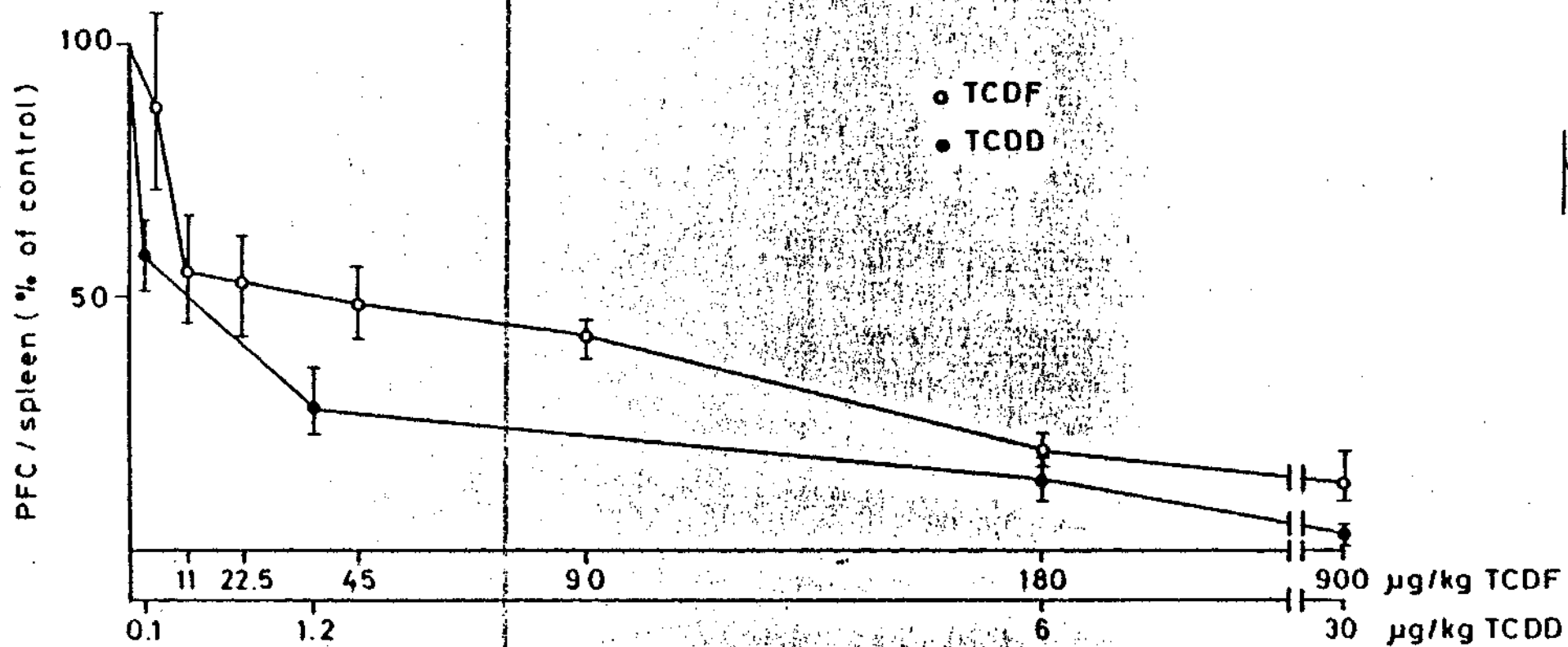


Fig. 1

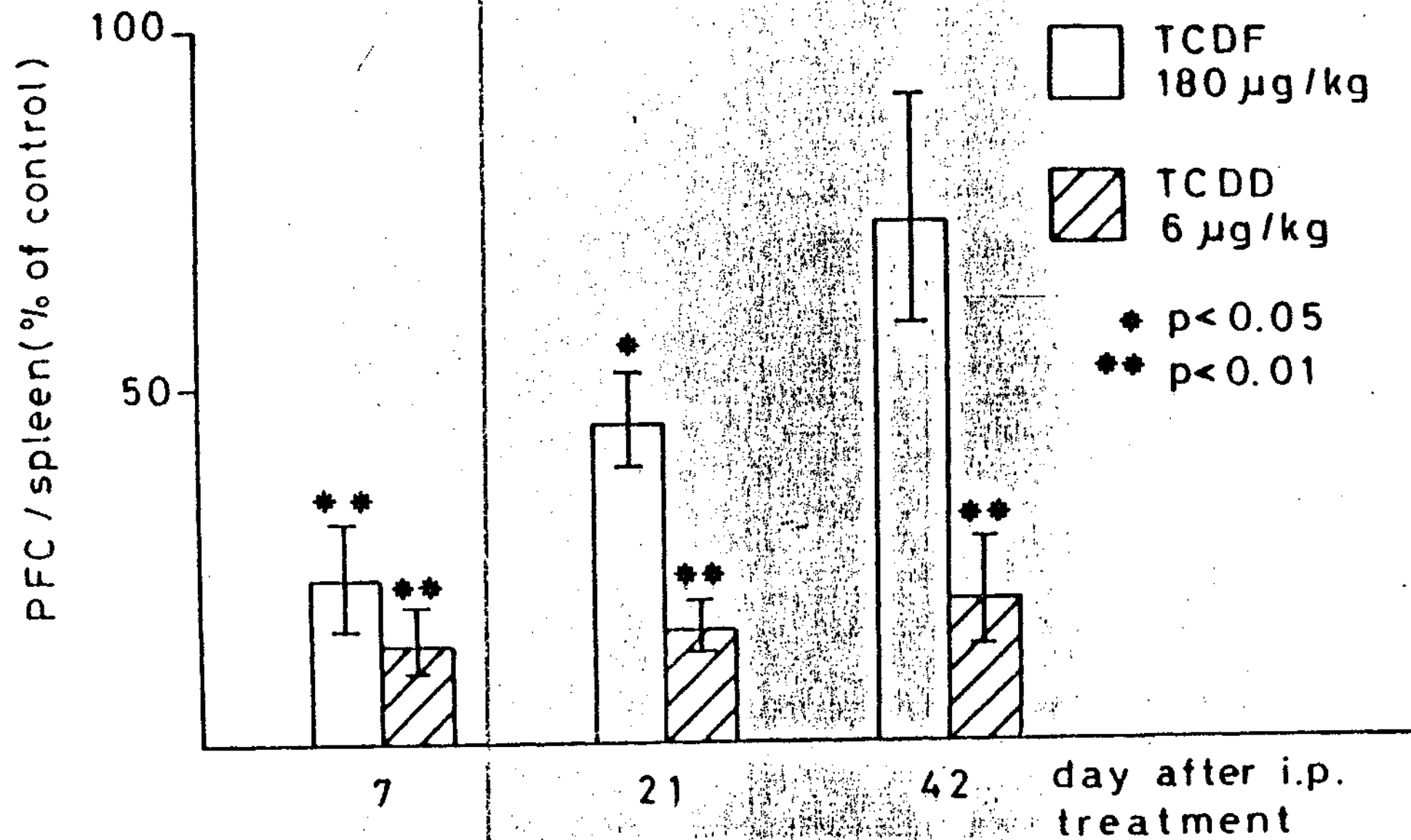


Fig. 2

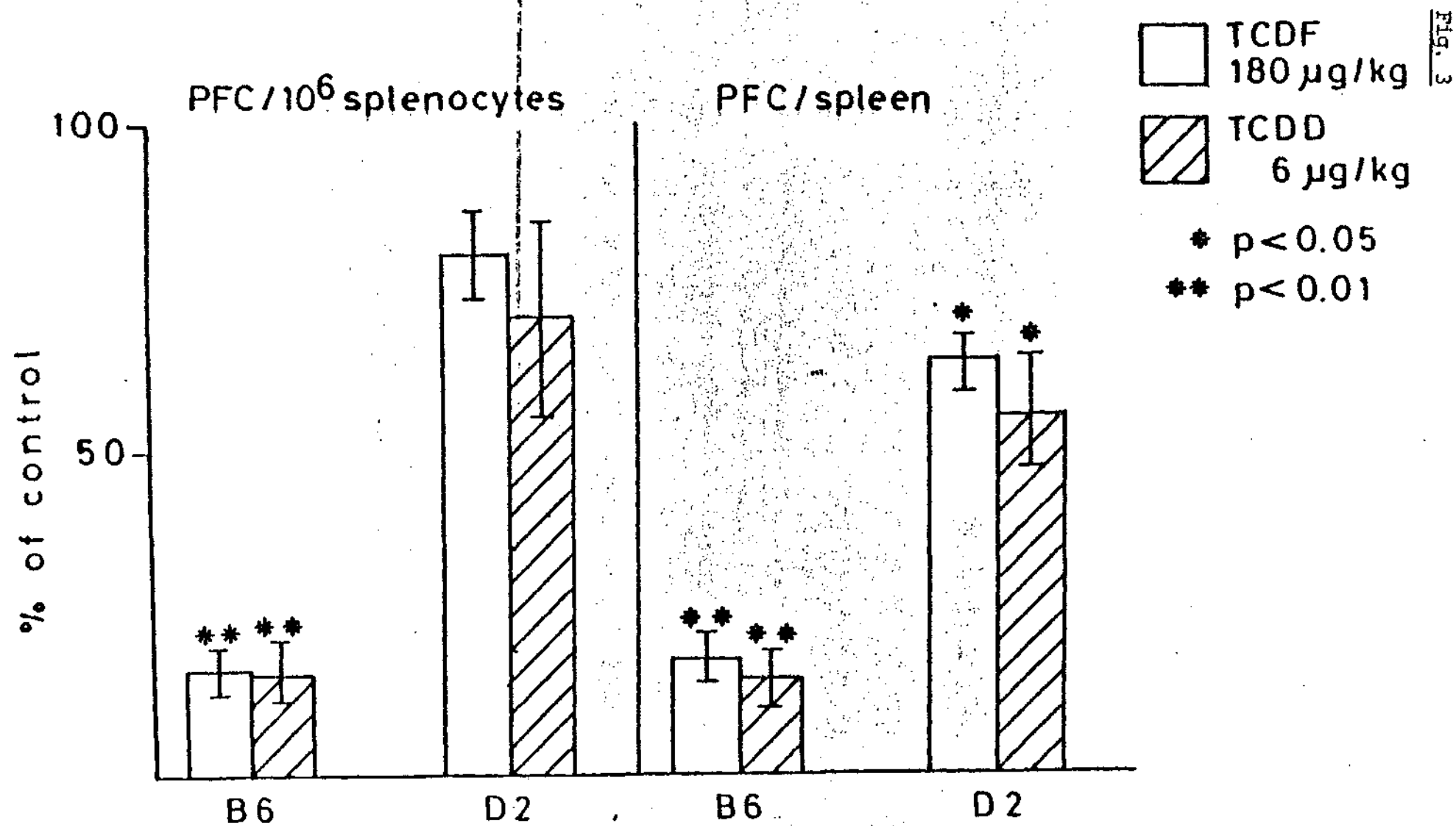


Table 1 - Effect of TCDF on body weight and lymphoid organs in C57Bl/6 mice

TCDF µg/kg	Route of admin.	Body weight (g)	Thymus weight (mg)	Spleen cells (x10 <sup>6</sup> )	P E C (x10 <sup>6</sup> )	Macrophages (x10 <sup>6</sup> )
0	i.p.	24.1±0.6	57.3±5.4	136.9±13.8		
22.5	"	25.8±1.0	57.0±3.0	111.8±6.5		
45	"	24.6±0.7	62.7±2.8	107.7±4.1 <sup>x</sup>		
90	"	24.2±0.8	55.1±5.1	120.7±7.5		
0	"	27.3±0.7	42.8±1.4	129.9±20.3	7.6±1.7	3.6±0.9
180	"	26.7±1.3	30.5±2.5 <sup>xx</sup>	74.9±8.3 <sup>xx</sup>	11.0±1.4	6.0±1.1
900	"	25.7±1.7	13.5±1.6 <sup>xx</sup>	64.4±7.2 <sup>xx</sup>	11.5±2.6	5.4±0.6
0	p.o.	28.8±1.0	44.7±1.4	143.1±26.5	4.2±0.6	1.9±0.2
180	"	26.2±0.3	37.8±2.2 <sup>xx</sup>	89.3±9.6 <sup>xx</sup>	3.6±0.4	1.8±0.2
900	"	25.1±1.3	27.8±2.9 <sup>xx</sup>	62.4±9.0 <sup>xx</sup>	4.2±0.7	1.9±0.2

TCDF was given 12 days before testing.

<sup>x</sup>p < 0.05

<sup>xx</sup>p < 0.01

PEC = Peritoneal exudate cells.

Table 2 - Immunosuppressive effect of TCDF given by different routes.

TCDF (pg/kg)	Route	PFC/ $10^6$ splenocytes	PFC/spleen	% of control
0	i.p.	206 (181-234)	24795 (20890-29428)	100
180	"	75 <sup>xx</sup> (52-107)	5692 <sup>xx</sup> (4159-7790)	23
900	"	62 <sup>xx</sup> (47-81)	3648 <sup>xx</sup> (1419-4037)	15
0	p.o.	336 (308-365)	48334 (35778-65297)	100
180	"	123 <sup>x</sup> (84-181)	10323 <sup>x</sup> (7716-13811)	21
900	"	111 <sup>x</sup> (70-175)	6207 <sup>xx</sup> (4120-9352)	13

TCDF was given on day -7;  $4 \times 10^8$  SREBC were injected i.p. on day 0 and test was performed on day +5.

x p < 0.05

xx p < 0.01

Logarithmic transformation of PFC values was done before statistical analysis. Results are antilogarithms of the means ( $\pm$  S.E.).

Table 3 - Effect of TCDD and TCDF on body and thymus weight in different mouse strains.

Strain	TCDD ( $\mu\text{g/kg}$ )	TCDF ( $\mu\text{g/kg}$ )	Body weight	Thymus weight (% of control)
B6	0		25.9 $\pm$ 0.5	64.3 $\pm$ 2.9 (100)
	6		24.5 $\pm$ 0.4	45.0 $\pm$ 4.3 <sup>x</sup> (70)
B6		0	21.4 $\pm$ 0.9	31.2 $\pm$ 3.0 (100)
		180	22.1 $\pm$ 0.9	18.6 $\pm$ 4.6 <sup>x</sup> (60)
D2	0		21.1 $\pm$ 0.5	27.7 $\pm$ 2.2 (100)
	6		22.7 $\pm$ 0.8	23.4 $\pm$ 3.5 (84)
D2		0	25.8 $\pm$ 0.5	37.6 $\pm$ 3.0 (100)
		180	26.3 $\pm$ 0.5	32.0 $\pm$ 2.4 (85)

TCDF and TCDD were given i.p. 12 days before evaluation

<sup>x</sup><sub>p</sub> < 0.05



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Ist Dermosyphilopathic Clinic of the University of Milan

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THE ULTRAMICROSCOPIC STRUCTURE OF THE  
COMEDONE IN CHLORACNE

Milan July 1979

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# The Ultramicroscopic Structure of the Comedone in Chloracne

## ABSTRACT

The ultramicroscopic study of comedones extracted from several children of the Seveso area affected with chloracne has brought to light a number of ultrastructural features which distinguish these comedones from those observed in acne vulgaris and which are ascribable to the presence in the pilary orifice of an active keratinizing factor. In view of the present findings and the associated clinical and epidemiological data, the authors conclude that there is evidence to support the hypothesis of a direct relationship between the formation of this particular type of comedone and TCDD contamination.

## The Ultramicroscopic Structure of the Comedone in Chloracne

The most late-appearing and specific dermatological pathology observed in the Seveso area following the ICMESA accident of July 10, 1979 was chloracne from TCDD. A large number of inhabitants primarily school-age children, were affected in the months following the disaster.

This chloracne was observed to have many of the same features as that commonly caused by other cyclically structured chlorates used in industrial processing. Various cases of chloracne resulting from dioxin poisoning are described in the literature.

Although the clinical picture of TCDD chloracne does not differ substantially from that of other types, several particular characteristics are of note:

- the rather frequent occurrence of corneal cysts on the eyelids and eyebrows;
- the initial and preferred location of comedones in the upper lateral cheek area;
- the relative dryness of the comedones;
- a slight turgidity near the follicle and sebaceous glands in the initial phase;
- the relatively slow formation and elimination of the comedone.

The comedone which fills the follicular orifice has been amply investigated in clinical and experimental contexts. These studies have clarified many etiological and pathogenetic aspects of the clinical course of acne; however, no data have thus far been available on the ultra-

structure of the chloracne comedone arising from TCDD; thus, an ultramicroscopic study was carried out on comedones extracted from the skin of subjects exposed to dioxin in Seveso and the surrounding area.

Due to the relative scarcity of examinable material, most of the observations were made with ultramicroscopic equipment, especially the electron microscope.

#### Present state of knowledge on the comedone

In order to afford the reader a better understanding of the present study, the authors have deemed it useful to provide a background of current knowledge on the nature, formation and structure of the comedone, a small clinical manifestation which is at the root of many widespread human pathologies.

The pilo-sebaceous follicle - the organelle in which the whole pathology of the comedone is enacted - is a complex, harmoniously designed structure. It is basically made up of several folds in the epidermis, an organ which has different structures and performs different functions at different levels.

Figures 1 and 2 offer a schematic illustration of the developmental stages of the hair follicle in the fetus.

Figure 3 shows the entire apparatus of the sebaceous glands and hair follicle, while Figure 4 illustrates the normal behavior of the follicular orifice. In Figure 5 the orifice is undergoing keratinization, giving rise to the formation of a comedone.

1. The pilary bulb (2) consists of a mass of cells which are stimulated by a papilla of connective tissue richly endowed with capillaries, nerve, cells, and mucopolysaccharides.

The cells of the bulb proliferate rapidly and line up in concentric layers around the hair, guiding its maturation and differentiation.

2. The hair (3) is made up of keratinized horny cells.

3. The follicular walls (8) are actually a fold or hollow of the epidermis (4) which continues up to the outer part of the bulb and unites with it.

4. The sebaceous gland consists of a localized differentiation of the epithelium. At this site, instead of explicating its usual keratinizing function, the epithelium gives rise to a fatty substance which colliquates and forms sebum. The sebum is excreted from the follicle and coats the surrounding skin surfaces.

5. The follicle widens in proximity to the epidermal surface to form an orifice. The structure of the orifice is identical to that of the epidermis.

We will now turn our attention to the orifice, since it is here that the comedone arises and evolves.

If the walls of the follicular orifice were to behave like the rest of the epidermis, of which in fact they are a continuation, the orifice would soon be hermetically sealed by a layer of horny cells. Figure 4 demonstrates that the protoplasm of the basal cell does not undergo the normal process of keratinization which in the epidermis gives rise to the horny layer (5). Rather, the protoplasm disin

tegrates and colliquates (7), leaving a flat, empty corneal shell (8) which falls into the orifice (6) and is subsequently expelled along with the sebum.

As a result of this peculiar behavior, the follicular orifice remains open and is thus able to eliminate elements of both internal and external origin (fats, hair fragments, horny structures, smog, dust bacteria, fungi, chemical compounds, parasites, etc.).

Following puberty, at which time the sebaceous gland undergoes intense functional development, the contents of the follicular orifice may thicken from evaporation or aging. A compact mass may then form and take on the clinical appearance of a comedone. This, however, is a "false" comedone, made up of thickened sebum and particles of other matter trapped in the sebum. The marks of the false comedone are:

- 1) it can be quite easily expelled;
- 2) it has a yellowish, ceruminous appearance and a soft, crumbly texture;
- 3) it is easily crushed between two microscope slides.

When particular occupational, hormonal or toxic factors are involved, this characteristic "physiological" behavior is disturbed and the orifice wall pathologically assumes a keratinizing function identical to that of the epidermal surface. The walls of the orifice generate horny layers which instead of being eliminated join to form a tight web that finally occludes the orifice completely (cf. Fig. 5). This is the "true" comedone, a compact, pearly-white horny structure which is usually very difficult to expel since the orifice often has a smaller diameter at the top than underneath.

In between these two contrasting types of comedone are both transient and permanent "intermediate" types, arising in response to keratinizing stimuli which act for varying lengths of time on the walls of the follicular orifice. The inflammatory pathology of adolescent acne is related to the "true" comedone; however, with few exceptions, the classical inflammations and suppurations of adolescent acne are caused by impregnation of the tissues with a particular fatty substance, seborrhea.

In most cases, the comedone which results from exogenous or endogenous occupational causes (mechanics' contact with mineral oil, lathe workers' cuts, contact with industrial chlorates) is a false comedone. Thus, except in young, seborrheic subjects, occupational acne usually runs its course without the usual phenomenology associated with adolescent acne (granulomas, it is hoped that this brief introduction will facilitate understanding of the study proper, the purpose of which is (1) to examine the nature, structure and characteristics of the comedone observed in subjects of the Seveso area affected with chloracne from TCDD, and (2) to interpret the significance of these findings.

The clinical course of chloracne observed especially in elementary school children (5-11 years of age) is associated, for reasons given elsewhere, with the action of dioxin present in the environment. Once introduced into the organism, dioxin is eliminated through the follicles.

This mechanism is similar to that observed in chloracne resulting from the inhalation or ingestion of volatile and non-volatile chlorates used in industrial processes; these,



too, are eliminated through the sebaceous glands.

The attribution of the chloracne observed in school-age children of Seveso and neighboring communities to the elimination of TCDD is supported by the following facts:

1. Numerous outbreaks of comedones occurred following the industrial accident, whereas none were observed previously in the same population, even among ICMESSA workers.
2. A much higher percentage of pathological cases was observed in the contaminated zones than in nearby residential areas only minimally affected by the contamination.
3. Chloracne affected primarily those subjects who came in to immediate contact with the toxic cloud or, thereafter, with contaminated soil.
4. The symptoms had the particular characteristics noted earlier.

Chlorates are known to prefer certain excretion sites. In the chloracne victims of Seveso, particular parts of the face were unaffected by comedones, despite an equal atmospheric exposure to TCDD. This, in addition to the symmetrical manifestations of the dermatoses, tends to demonstrate that a chlorate, in this case TCDD, is being eliminated through the follicles, probably via the sebum.

The comedones which appeared in children affected with chloracne from TCDD do not, then, differ significantly from those observed in workers affected with chloracne from industrial products, though several morphological and developmental characteristics distinguish the clinical course of the former to a certain extent.