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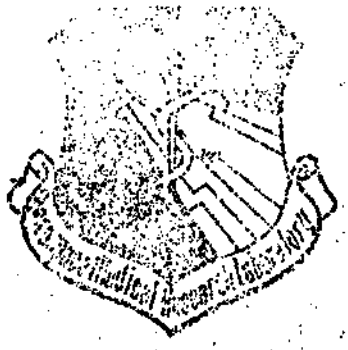

THE TOXICITY OF PERFLUORO-N-DECANOIC ACID AND 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN IN L5178Y MOUSE LYMPHOMA CELLS

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FOR THE COMMANDER

ROGER C. INMAN, Colonel, USAF
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Perfluoro-n-decanoic acid (PFDA) causes toxic sequelae in vivo very similar to those caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The toxicity of these two compounds, several other polyfluorinated fatty acids, and corresponding hydrogenated fatty acids have been studied in vitro in L5178Y mouse lymphoma cells. Below concentrations which cause cell lysis (>500 μg/ml), PFDA did not affect suspension growth. After 24 hr treatment with concentrations between 1 and 100 μg/ml treated cells no longer grew into clones when plated in semi-soft agar. This impairment of clone-forming ability was reversible after growth of

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treated cells in fresh medium for 36 hr. Perfluoro-n-octanoic acid did not impair clone-forming ability at any concentration; and neither did the straight-chain hydrogenated fatty acid analogs. All polyfluorinated acids tested (either perfluorinated or ω-hydro-analogs) with chain length 9 or greater caused impairment of clone-forming ability after treatment with concentrations that were non-toxic in suspension. TCDD (highest dose, 0.5 μg/ml) had no effect on growth in suspension. After 48 hr treatment with TCDD concentrations of 0.01 μg/ml or greater, plated cells formed clones with altered morphology. These clones were less discrete, lacking a clearly defined boundary. The effect on clone morphology required 36 hr treatment of cells with TCDD in suspension and was reversible following 48 hr growth in fresh medium. Cell division time in suspension was 10-12 hrs and was unaffected by PFDA or TCDD. In vivo PFDA treatment altered erythrocyte fragility in rats. It is suggested that the toxicity of PFDA and TCDD in vivo and in L5178Y cells in vitro may be due to an ability of these chemicals to interfere with normal structure and/or function of biological membranes.

PREFACE

This research was performed in the Biochemical Toxicology Branch, Toxic Hazards Division, Air Force Aerospace Medical Research Laboratory from January 1980 through December 1981. It was performed in support of Task 2312V1, "Toxicological Mechanisms of Air Force Chemicals and Materials;" Work Unit 2312V118, "Effects of Air Force Propellants and Chemicals on Metabolic Mechanisms." Portions of this work were presented at the 21st Annual Meeting of the Society of Toxicology, Boston, Massachusetts, 22-26 February 1982.

INTRODUCTION

Perfluorinated fatty acids, perfluorinated sulfonic acids, and appropriate derivatives are used commercially in numerous applications which take advantage of their exceptional surfactant properties and extreme chemical and thermal stability (Guenthner and Victor, 1962). Most commercially important derivatives are based on perfluoroalkyl chain lengths of 5 to 7. The acute and subchronic toxicity of ammonium perfluoro-n-octanoate (PFOA) has been described in detail in both rats and rhesus monkeys (Griffith and Long, 1980). Less is known of the toxicity of longer chain analogs.

In an abstract Andersen et al. (1981) described the acute toxicity of perfluoro-n-decanoic acid (PFDA; nonadecafluoro-n-decanoic acid; $C_{10}F_{19}O_2H$) in a variety of rodent species. This acid was significantly more toxic than PFOA and its toxicity differed both quantitatively and qualitatively from that of the shorter chain analog. Toxic signs and target organs for PFDA were similar to those seen with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The single dose oral LD_{50} - 30 days of PFDA in male rats was about 50 mg/kg and rats intubated with 90 mg/kg lost nearly 50% of their initial body weight before dying two to three weeks after intubation. As does TCDD, PFDA caused severe thymic atrophy.

As part of a comparison of the biological effects of PFDA and TCDD, we have evaluated the toxicity of these chemicals in several isolated cell systems. In part, this paper describes effects of various polyfluorinated fatty acids, hydrogenated fatty acids, and TCDD on growth characteristics of L5178Y mouse lymphoma cells, a T-cell derived lymphoma (Muller et al., 1981), which grows both in suspension and in semi-soft agar. A T-cell lymphoma was used because T-lymphocytes appear to be targets of PFDA and TCDD toxicity in rodents. This conclusion was based on the marked thymic cortical atrophy noted in animals treated with either of these chemicals. L5178Y cells are commonly used for mutagenicity testing and the mutagenic potential of these chemicals in L5178Y cells is reported elsewhere (Rogers et al., 1982). In addition, limited results of osmotic fragility studies of erythrocytes from rats treated with PFDA are described in an attempt to relate altered osmotic fragilities to the effects of PFDA on L5178Y cells.

MATERIALS AND METHODS

L5178Y Mouse Lymphoma Cells: L5178Y cells were originally obtained from Dr. C. F. Arlett, MRC, Cell Mutation Unit, Brighton, England. They were routinely screened for mycoplasma contamination. The routine methods for maintenance of L5178Y cells and the soft agar cloning technique were as described elsewhere (Cole and Arlett, 1976), except that McCoy's 5A medium (supplemented with penicillin, streptomycin, sodium pyruvate, and 10% horse serum) was used instead of Fischer's medium. For toxicity experiments, L5178Y cells were treated for 24 hrs with doses of PFDA ranging from 0.01 µg/ml to 1 mg/ml, or for 48 hrs with doses of TCDD ranging from 0.001 µg/ml to 0.50 µg/ml. At the end of the treatment period, cells were centrifuged, washed in McCoy's 5A medium and resuspended in McCoy's 5A containing 20% horse serum. Cells were plated for growth in soft agar, and plates were examined for clones after 9-10 days incubation in a humidified CO_2

incubator. Horse serum was obtained from Gibco-Biocult. Penicillin, streptomycin, and sodium pyruvate were obtained from Sigma.

Chemicals: Fatty acids (all > 99% pure)[1], perfluoro-n-decanoic acid (>98%)[1], perfluoro-n-octanoic acid (>96%)[1], 11-H eicosafluoro-n-undecanoic acid (97-99%)[2], and 9-H hexadecafluoro-n-nonanoic acid[3] were obtained commercially. The latter two compounds contain a single hydrogen at the omega position. Perfluoro-n-dodecanoic acid[4] (71% $C_{11}F_{23}CO_2H$; 3% $C_{10}F_{21}CO_2H$; 2% $C_9F_{19}CO_2H$; remainder unidentified, nonfunctional fluorocarbon) and TCDD were gifts[5]. For L5178Y studies, TCDD was dissolved in acetone and the fatty acids and fluorinated analogs were dissolved in dimethylsulfoxide except perfluorinated dodecanoic was also dissolved in acetone.

Osmotic Fragility: Male Fischer 344 rats (200-300 g) were treated ip with 50 mg PFDA/kg. Propylene glycol:water (50:50 v/v) was used as diluent with a final dosing volume of 2 ml/kg. Treated and diluent control rats in groups of four to five were killed at various times after injection. Blood was drawn from the inferior vena cava after opening the abdomen of anesthetized rats and erythrocytes harvested by centrifugation. Osmotic fragility was determined as described in Dacie and Lewis (1963). Curves were constructed for hemolysis at 10 saline concentrations between 0.25 and 0.85%. Data presented are percent hemolysis at a single intermediate saline concentration, 0.4%.

RESULTS

Fatty Acids: PFDA had little effect on L5178Y suspension growth at concentrations below 100 µg/ml (Fig. 1). At concentrations of 500 µg/ml or above, cells were dissolved by the surfactant action of the acid and neither cells nor debris were visible in suspensions at these concentrations. In comparison to the dose-response curve for suspension growth, the curve for clone-forming ability was shifted some 2.5 log units to the left: the ED_{50}-24 hr for impairing clone-forming ability was approximately 3×10^{-1} µg/ml. To our knowledge, this ability - dissociating the markers of suspension growth and clone-forming ability in these transformed cells - has not been reported for any other chemical. Perfluorinated-n-dodecanoic and 11-H-eicosafluoro-n-undecanoic acid caused a similar displacement of the two dose response curves (Table 1). On the other hand, PFOA which was slightly less toxic to cells in suspension than was PFDA did not show the differential toxicity with respect to suspension and clonal growth. The ω-H-hexadecafluoro-n-nonanoic acid displaced the dose response curves for suspension and clone forming ability, but the displacement was less than that seen with PFDA (Table 1). With hydrogenated fatty acid analogs from C_8 to C_{11}, toxicity was equal both in suspension and in agar (Table 2).

[1] Aldrich Chemical Company, Milwaukee, WI 53233.
[2] PCR Research Chemicals, Inc., Gainesville, FL 32602.
[3] Alfred Bader Library of Rare Chemicals, Division of Aldrich Chemical Company, Milwaukee, WI 53233.
[4] Commercial Chemicals Division 3M, 3M Center, St Paul, MN 55144.
[5] Dow Chemical USA, Midland, MI 46460.

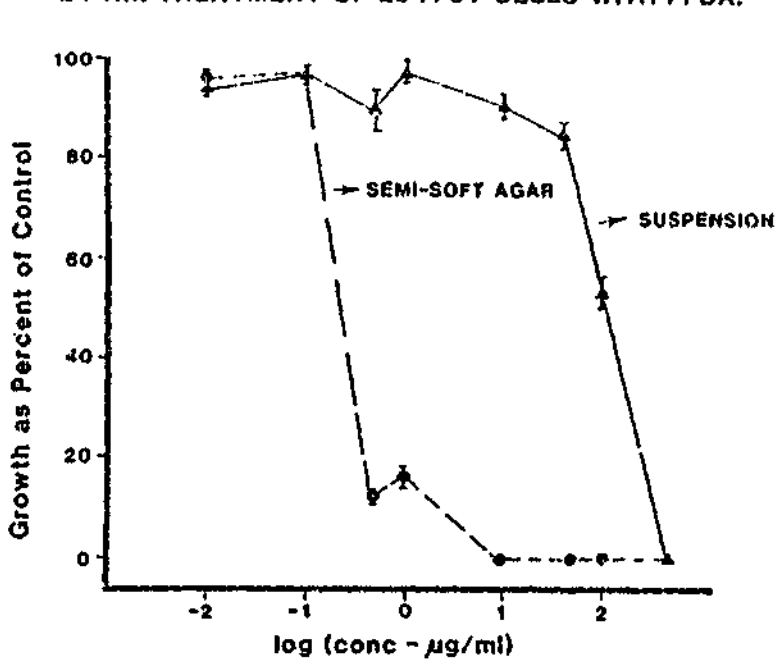


Figure 1. Toxicity of PFDA in L5178Y Cells. L5178Y cells were grown for 24 hr in the presence of varying amounts of PFDA (x-axis). Triangles are total growth in suspension as percent of control growth in the absence of PFDA. Aliquots of the cells treated with different concentrations were plated and grown for 8 to 10 days on semi-soft agar. Circles are percent of plated cells which gave rise to clones relative to similar values for control cells. Data points are mean and standard errors (n = 3-7).

Table 1

The Effects of Various Polyfluorinated Fatty Acids on Growth of L5178Y Mouse Lymphoma Cells in Suspension and on their Clone Forming Ability in Semi-Soft Agar

Dose	Perfluoro-n-octanoic Acid		9-H Hexadecafluoro-n-nonanoic Acid		Perfluoro-n-decanoic Acid		11-H Eicosafluoro-n-undecanoic Acid		Perfluoro-n-dodecanoic Acid	
	Suspension	Agar	Suspension	Agar	Suspension	Agar	Suspension	Agar	Suspension	Agar
(µg/ml)	(% Control)		(% Control)		(% Control)		(% Control)		(% Control)	
0.01	94[a]	98	94	97	94 ± 2[b]	96	96	100	87	97
0.1	100	96	83	95	97 ± 1.4[c]	97 ± 0.7	78	90	78	87
0.5					89 ± 4.7[b]	12 ± 1.1				
1	89	98	94	95	97 ± 1.1[b]	16 ± 1.9	75	11	91	25
10	89	98	94	89	90 ± 2.2[b]	0	82	0	72	0
50	76	90	92	89	84 ± 2.3[b]	0	82	0	75	0
100	76	90	83	0	53 ± 3.6[b]	0	82	0	72	0
500	21	21	--[d]	--	---[d]	--	--[d]	--	--[d]	--
1000	--[d]	--	--[d]	--	---[d]	--	--[d]	--	--[d]	--

[a] Result of single experiment. Numbers in each column are growth as percent of growth of control cells.
[b] Mean ± standard error (n = 3).
[c] Mean ± standard error (n = 7).
[d] These concentrations dissolved cells in suspension.

Table 7

The Effect of Various Fatty Acids on Growth of L5178Y Mouse Lymphoma Cells in Suspension and on their Clone Forming Ability in Semi-Soft Agar

Dose	Nonanoic Acid		Decanoic Acid		Undecanoic Acid	
	Suspension	Agar	Suspension	Agar	Suspension	Agar
(μg/ml)	(% Control)		(% Control)		(% Control)	
0.01	85[a]	98	106	96	95	96
0.1	82	98	94	94	85	93
1	92	98	100	93	75	96
10	90	94	84	90	82	93
50	85	98	75	82	82	86
100	72	88	63	63	42	44
500	33	26	--[b]	--	--[b]	--
1000	--[b]	--	--[b]	--	--[b]	--

[a] Numbers in both columns are growth as percent of growth of control cells.
[b] These concentrations dissolved cells in suspension.

Time to Effect and Reversibility: Cell division time for L5178Y cells in suspension under growth conditions used in this study was 10 to 12 hours. An experiment was performed to see if cells required a period of treatment with PFDA before diminished clone-forming ability could be observed. Cells were grown in suspension containing 0.5 μg PFDA/ml and removed at various times for plating to observe loss of clone-forming ability (Fig. 2a). There was a lag of 8 hr before any appreciable effect was observed and the time of treatment required to reduce plating efficiency to 50% of control was about 12 hr, i.e., one cell generation.

Cells were also grown for 24 hr in the presence of 0.5 μg of PFDA/ml harvested by centrifugation and washed in fresh growth medium. These treated cells were resuspended for growth in fresh, uncontaminated medium and aliquots withdrawn after various times for plating (Fig. 2b). The decreased plating efficiency was reversible, but recovery was more prolonged than the time required to induce the diminished clone-forming ability. The time of growth in fresh medium necessary to restore 50% plating efficiency was nearly 36 hr, or about three cell generations. Cell division time of L5178Y cells in suspension was unaffected by pretreatment with 0.5 μg PFDA/ml.

Dioxin: In L5178Y cells TCDD did not dissociate growth in suspension from growth in soft agar at any concentration tested, up to 0.5 μg/ml. However, the morphology of the clones obtained after treating cells in suspension with concentrations of TCDD between 0.01 and 0.5 μg/ml, was markedly different from controls (Fig. 3). Instead of the well-circumscribed, circular clusters of control clones, those clones formed after dioxin-treatment were less-discrete and lacked a well-defined border. After growing cells in suspension for 46 hr in the presence of 0.005 μg TCDD/ml,

TIME TO RESPONSE/TIME TO RECOVERY OF L5178Y CELLS TREATED WITH PFDA.

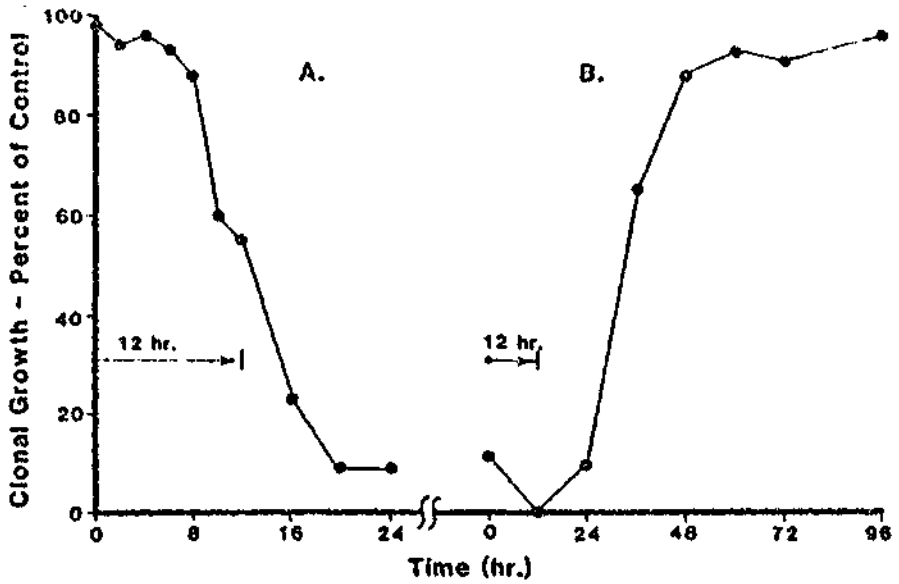
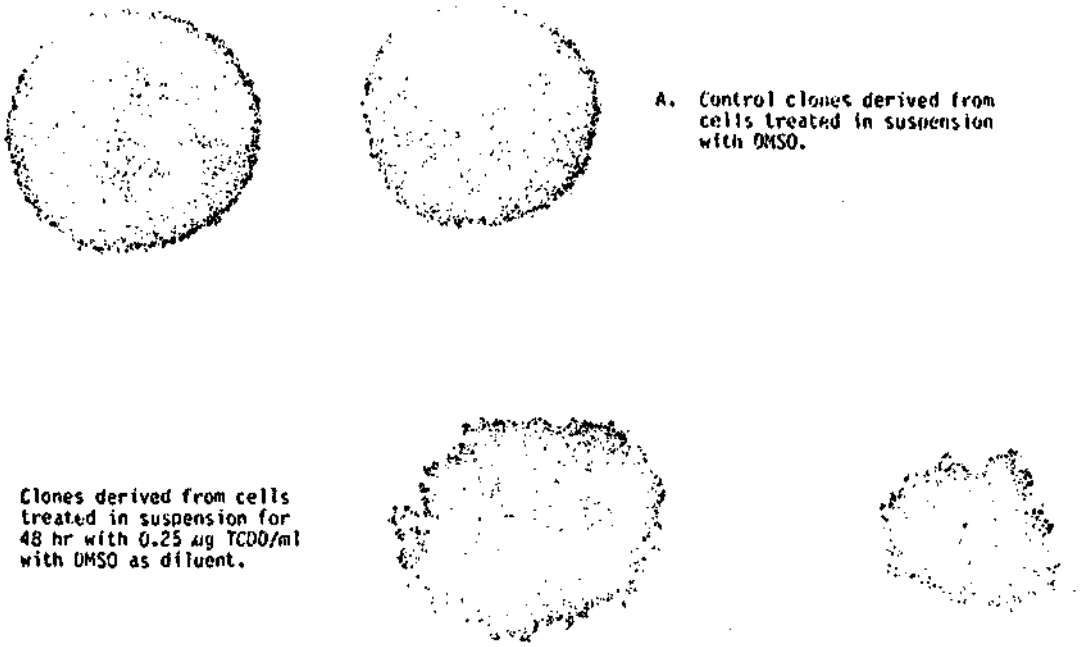


Figure 2. Time course of impairment and recovery of cloning ability in L5178Y cells treated with PFDA in suspension. A: Time to effect: cells were grown for various lengths of time (x-axis) in suspension in a medium containing 0.5 µg PFDA/ml and plated in semi-soft agar. Growth is expressed as percent of plated cells forming clones after treatment relative to percent of untreated cells which give rise to clones. B: Time to recovery: cells were treated in suspension with 0.5 µg PFDA/ml for 24 hr, and harvested by centrifugation. Aliquots were removed and grown in fresh, uncontaminated medium for various lengths of time (x-axis). Cells were then plated to observe recovery of the ability to form clones.

all clones formed after plating were normal; at 0.01 µg/ml, most clones formed were abnormal; and by 0.5 µg/ml, all clones had altered morphology. By inspection of the plates, the ED_{50}, that is the concentration of dioxin required to produce alterations affecting 50% of the formed clones when cells were initially maintained in suspension with dioxin for 48 hr before plating, was about 0.01 µg/ml, i.e., about 3×10^{-8}M.

In time-course experiments analogous to those in Fig. 2, but conducted with 0.01 µg dioxin/ml, the time of treatment in suspension required to produce 50% of maximum response in altering clone morphology was about 36 hr. A time to recovery of normal growth characteristics was also estimated for cells grown initially for 48 hr in the presence of 0.01 µg TCDD/ml. The time of growth in fresh medium required to give a 50% return to normal clonal morphology was about 48 hr. As noted with PFDA, effects on clone growth were reversible, but recovery and expression times for the effects were longer with TCDD than with PFDA.

Red Blood Cell Fragility: Rat red blood cells were obtained from rats killed at various times after ip injection of 50 mg PFDA/kg. There was increased resistance to hemolysis after treatment with PFDA (Fig. 4) and the time course of decreased fragility was similar to the time course of weight loss in treated rats (Andersen et al., 1981).



A. Control clones derived from cells treated in suspension with DMSO.

B. Clones derived from cells treated in suspension for 48 hr with 0.25 μg TCDD/ml with DMSO as diluent.

Figure 3. Altered clone morphology after treating L5178Y cells in suspension with TCDD.

DISCUSSION

Knutson and Poland (1980) studied the effects of TCDD on 23 cultured cell types and found no toxicity in any of these mammalian cell lines at treatment concentrations of up to 10^{-7}M and contact times of up to two weeks. Markers for toxicity included (1) alterations in the morphology of cells or the cell cultures, (2) percentage viable cells, and (3) growth rate. Among the 23 cell lines were five lymphoid cell types derived from thymic cortex - three were murine and two were virally transformed human leukocytes. All these cell types were tested for growth in suspension and cell viability by trypan blue exclusion. Beatty et al. (1975) found that TCDD had no effect on growth or morphology of normal human lymphocytes in suspension. Our results are similar to the extent that TCDD did not affect growth or cell viability of L5178Y cells in suspension. The altered growth characteristics observed in this paper are more subtle and only apparent when cells are grown in semi-soft agar, where they are constrained to grow in close proximity to each other. The concentration dependence of the effect with TCDD is such that a 48 hr treatment with 0.01 μg/ml (i.e., about 3×10^{-8}M TCDD) causes the effect in most of the treated cells. This concentration is reasonable for physiological significance since the mouse LD_{50} is about 300 μg TCDD/kg, or about 1 μmoles/kg (McConnell et al., 1978).

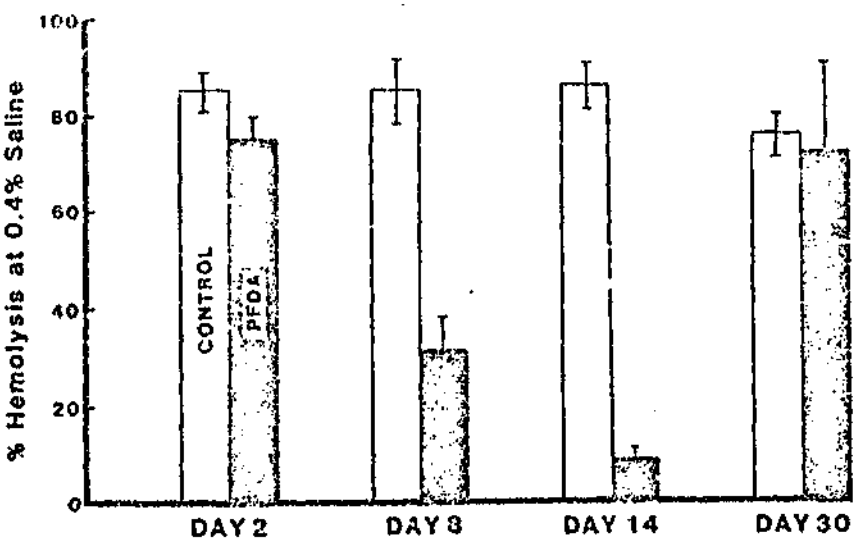


Figure 4. Relative osmotic fragilities of red blood cells from rats injected ip with 50 mg PFDA/kg and killed at various times after injection. Data are mean and standard deviation (n = 4-5). From the overall curves with 10 salt concentrations, the concentration at which 50% hemolysis occurred was 0.43, 0.38, 0.34, and 0.43%, respectively, in treated rats at 2, 8, 14, and 30 days. Control groups at these sampling t ·s had 50% hemolysis at 0.45, 0.44, 0.45, and 0.43%, respectively.

Alterations in clone morphology seen after TCDD are striking, but estimations of concentration dependence are essentially qualitative, i.e., the percentage of abnormal clones is estimated by inspection and making a distinction between normal and slightly abnormal clones is difficult. We have maintained a restrictive definition of what constitutes an abnormal clone and scoring was done solely by Dr. A. M. Rogers. For these reasons, the estimated ED_{50} for the effects with TCDD are probably high. More quantitative determinations of these TCDD dose response curves await determination of the biochemical basis of the altered morphology and methods to unequivocally identify altered clonal units.

With PFDA, results are readily quantified since treated cells no longer proliferate in semi-soft agar. The ED_{50} - 24 hr for the loss of clone-forming ability was 0.3 µg/ml (i.e., about 6×10^{-7}M); this contrasts to a single dose LD_{50} in mice of about 100-150 mg PFDA/kg or 0.2-0.3 mmoles/kg (Andersen et al., 1981; Van Rafelghem and Andersen, unpublished experiments, 1981).

With the polyfluorinated acids examined, this toxicity is present with acids of chain length greater than 8. The differences in single cell toxicity between the fluorinated octanoic and decanoic acids are striking, but consistent with the different acute toxicity reported for these two acids in rats. The hydrogenated fatty acids are without differential effect on clone-forming ability of L5178Y cells. In terms of cell lysis, expressed

as toxicity in suspension, hydrogenated and polyfluorinated fatty acids were about equipotent (Tables 1 and 2).

The molecular basis of the impairment of clone-forming ability is unknown. Subtle changes may have occurred in cell membranes to inhibit growth of cells when maintained in close contact. In this regard, the osmotic fragility results suggest a biological membrane more resistant to hyposmotic insult. Increased resistance can be due to a variety of causes, one of which is altered membrane composition (Kuiper et al., 1971). Preliminary studies in our laboratory have now shown that erythrocytes from PFDA-treated animals also show increased membrane fluidity (M. George and M. E. Andersen, unpublished studies, 1982), and that the total fatty acid composition of the liver lipid pool in these rats shows a dramatic shift toward increasing unsaturation, especially in the stearic to oleic acid ratio (Olsen et al., 1982). While indirect, these results suggest compositional and functional alterations in membranes subsequent to PFDA exposure in the rat in vivo.

There is no unifying hypothesis explaining the toxicity of TCDD and materials causing similar toxic effects, i.e., certain polyhalogenated biphenyls (Sleight et al., 1981; Biocca et al., 1961) and long-chain perfluorinated fatty acids of chain length 9 or above (Andersen et al., 1981). It may be that these various chemicals, including PFDA and TCDD, are toxic due to effects on cell membranes resulting in interference with cell-cell or cell-mediator interactions. These effects could either be direct or mediated by interference with some endogenous hormonal control of membrane composition/function. Toxicity would not be a result of cell necrosis or grossly visible organellar alterations, but from more subtle structural alterations of biomembranes and attendant disturbances in intercellular communication. This hypothesis is under active investigation in our laboratory.

The cell line used for this research was so-called TK $^{+/+}$ with regard to the gene locus for the enzyme thymidine kinase (TK). Our stock of these cells, brought to Dayton from England by Dr. Rogers, was destroyed during a malfunction of the deep freeze storage unit. We have not observed differential effects on suspension and clonal growth with L5178Y TK$^{+/-}$ cells - a cell line more commonly used in mutation research and, therefore, much more readily available. It appears that future work on this phenomenon will have to be restricted to the TK$^{+/+}$ cells.

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