

Toxicity of Ammonium Perfluorooctanoate in Male Cynomolgus Monkeys after Oral Dosing for 6 Months

John Butenhoff,^{*1} Giovanni Costa,[†] Cliff Elcombe,[‡] David Farrar,[§] Kristin Hansen,^{*} Hiroyuki Iwai,[¶] Reinhard Jung,^{||} Gerald Kennedy, Jr.,^{|||} Paul Lieder,^{*} Geary Olsen,^{*} and Peter Thomford^{||||}

^{*}3M, St. Paul, Minnesota 55144; [†]University of Verona, Verona 37134, Italy; [‡]University of Dundee, Dundee DD1 95Y, Scotland; [§]Ineos Chlor, Runcorn, Cheshire WA7 4JE, United Kingdom; [¶]Daikin, Osaka 566-8585, Japan; ^{||}Clariant, Sulzbach D-65840, Germany; ^{|||}DuPont, Newark, Delaware 19714; and ^{||||}Covance, Madison, Wisconsin 53704

Received February 2, 2002; accepted May 2, 2002

Ammonium perfluorooctanoate (APFO) is a processing aid in the production of fluoropolymers that has been shown to have a long half-life in human blood. To understand the potential toxicological response of primates, groups of male cynomolgus monkeys were given daily po (capsule) doses of either 0, 3, 10, or 30 (reduced to 20) mg/kg/day for 26 weeks. Two monkeys from each of the control and 10 mg/kg/day dose groups were observed for 90 days after the last dose. Clinical observations, clinical chemistry, determination of key hormones, gross and microscopic pathology, cell proliferation, peroxisomal proliferation, bile-acid determination, and serum and liver perfluorooctanoate (PFOA) concentrations were monitored. Toxicity, including weight loss and reduced food consumption, was noted early in the study at the 30 mg/kg/day dose; therefore, the dose was reduced to 20 mg/kg/day. The same signs of toxicity developed in 3 monkeys at 20 mg/kg/day, after which treatment of these monkeys was discontinued. One 30/20 mg/kg/day monkey developed the signs of toxicity noted above and a possible dosing injury, and this monkey was sacrificed *in extremis* on Day 29. A 3 mg/kg/day dose-group monkey was sacrificed *in extremis* on Day 137 for reasons not clearly related to APFO treatment. Dose-dependent increases in liver weight as a result of mitochondrial proliferation occurred in all APFO-treated groups. Histopathologic evidence of liver injury was not observed at either 3 or 10 mg/kg/day. Evidence of liver damage was seen in the monkey sacrificed in moribund condition at the highest dose. Body weights were decreased at 30/20 mg/kg. PFOA concentrations in serum and liver were highly variable, were not linearly proportional to dose, and cleared to background levels within 90 days after the last dose. A no observable effect level was not established in this study, and the low dose of 3 mg/kg/day was considered the lowest observable effect level based on increased liver weight and uncertainty as to the etiology leading to the moribund sacrifice of one low-dose monkey on Day 137. Other than those noted above, there were no APFO-related macroscopic or microscopic changes, changes in clinical chemistry, hormones, or urinalysis, or hematological effects. In particular, effects that have been associated with the development of pancreatic and testicular toxicity in rats were not observed in this study.

Key Words: ammonium perfluorooctanoate; repeated-dose toxicity; cynomolgus monkey; health effects; hepatotoxicity; APFO.

Ammonium perfluorooctanoate (APFO; C₇F₁₅COO⁻NH₄⁺, C.A.S. Registry number 3825-26-1) has been used as a processing aid in the production of fluoropolymers. Perfluorooctanoate (PFOA; C₇F₁₅COO⁻), the dissociation product of APFO, has been identified in blood samples from exposed workers and the general population (Belisle, 1981; Guy *et al.*, 1976; Hansen *et al.*, 2001; Olsen *et al.*, 1998, 2000; Ubel *et al.*, 1980).

Extensive research into the potential health risk of exposure to APFO was initiated in the late 1970s. Medical monitoring of employees involved in APFO production began in 1976, by measuring serum levels of organic fluorine and performing medical assessments (Ubel *et al.*, 1980). Since the early 1990s, serum concentrations of PFOA have been measured (Olsen *et al.*, 1998, 2000). Eighty percent of the workers had serum PFOA levels less than 6 ppm. Average serum concentrations have been approximately 2 ppm and individual values have ranged from undetectable to 114 ppm.

The potential association of APFO exposure with health effects in fluorochemical production workers has been studied in mortality and clinical studies. No excess cause-specific mortality has been associated with APFO exposure in this workforce (Alexander, unpublished report), and clinical indications of liver and hormonal function, including estradiol, testosterone, and cholecystokinin (CCK), have been normal (Olsen *et al.*, 1998, 2000).

APFO, once dissociated to form PFOA, is not metabolized further (Kuslikis *et al.*, 1992; Vanden Heuvel *et al.*, 1991). There are dramatic sex and species differences in elimination of PFOA (DuPont Haskell Laboratories, 1982; Johnson and Ober, 1980). In male rats, the elimination half-life is approximately 1 week, as opposed to female rats, which clear APFO rapidly with an elimination half-life close to 1 h. Recently developed data from an iv toxicokinetic study in male and female cynomolgus monkeys suggests that the terminal phase elimination half-lives in both sexes of the cynomolgus monkey are approximately 1 month (Butenhoff *et al.*, in preparation). PFOA has been demonstrated to have a long serum elimination half-life in workers (Ubel *et al.*, 1980), which based on recent

¹ To whom correspondence should be addressed at 3M Medical Department, Corporate Toxicology, 3M Center 220-2E-02, St. Paul, MN 55144. Fax: (651) 733-1773. E-mail: jlbutenhoff@mmm.com.

monitoring of retired fluorochemical workers, may be on the order of several years. In addition to urinary excretion, biliary excretion and reabsorption of PFOA are significant, as evidenced by the fact that whole-body elimination was significantly enhanced by cholestyramine administration in APFO-treated male rats (Johnson *et al.*, 1984).

Past dietary studies in rats and mice have demonstrated that the primary target organ for APFO-induced toxicity is the liver (Biegel *et al.*, 2001; Griffith and Long, 1980; Kennedy, 1987; Pastoor *et al.*, 1987). The hepatotoxicity manifests as increased liver weights, hepatocellular hypertrophy, liver degeneration, increases in plasma ALT and AST, necrosis of the liver, proliferation of smooth endoplasmic reticulum and peroxisomes, and hypolipidemia (Haughom and Spydevold, 1992; Intrasukri *et al.*, 1998; Kawashima *et al.*, 1995; Pastoor *et al.*, 1987; Permadi *et al.*, 1992, 1993). Many of these effects were demonstrated to be reversible when animals were provided with a recovery period. Deaths and weight loss also occurred in these studies, with 100% mortality occurring in both mice and rats at a dietary dose of 3000 ppm.

It is interesting to note that the only previous primate repeat-dose study did not identify the liver as a target organ (Griffith and Long, 1980). In this study, groups of two male and two female rhesus monkeys were treated with APFO by gastric intubation over a 90-day period at dose levels of either 0, 3, 30, or 100 mg/kg. There were no reported effects at 3 mg/kg/day. All monkeys in the 100 mg/kg/day dose group died prior to scheduled sacrifice. One male and both females died at 30 mg/kg prior to scheduled sacrifice, and males and females experienced reductions in body weight at 30 and 100 mg/kg.

APFO is included among several members of a structurally diverse group of compounds that induce peroxisome proliferation and tumors in rat liver, as well as extrahepatic tumors of the pancreas (acinar cells) and/or testis (Leydig cells; Biegel *et al.*, 2001; Cook *et al.*, 1999; Maloney and Waxman, 1999; Reddy and Rao, 1977; Riker Pharmaceuticals, 1987). In a chronic mechanistic dietary study, APFO produced this triad of tumors in rats but was considerably less potent than the model peroxisome proliferator, WY-14,643 (Biegel *et al.*, 2001). Liver tumors produced by peroxisome proliferating compounds may derive from the increased oxidative stress and cell proliferation that accompanies an increase in peroxisomes. However, the Leydig cell and pancreatic acinar cell tumors appear to result from mechanisms other than peroxisome proliferation. Specifically, it is currently thought that Leydig cell tumors in rats associated with peroxisome proliferators may result from the hyperplastic effect of sustained increases in estradiol as a result of induction of aromatase (Biegel *et al.*, 1995, 2001; Cook *et al.*, 1992; Liu *et al.*, 1996a,b). The mechanism for the production of pancreatic acinar cell hyperplasia and tumor formation by certain peroxisome proliferating compounds in rats is less clear. *In vitro* and *in vivo* experiments with WY-14,643 suggest that these exocrine pancreas tumors

may be the result of a mild, sustained increase in CCK due to cholestasis (Obourn *et al.*, 1997).

The occurrence of the triad of hepatocellular, pancreatic acinar cell, and Leydig cell tumors associated with peroxisome proliferation is likely to be species specific. The phenomenon of peroxisome proliferation is not uniform across all species. While rats and mice are particularly sensitive to this phenomenon, guinea pigs, cats, dogs, and primates (including humans), are predominantly nonresponsive (Cattley *et al.*, 1999; Kurata *et al.*, 1998; Pugh *et al.*, 2000). A number of compounds that produce Leydig cell tumors in rats fail to do so in mice (Clegg *et al.*, 1997; Cook *et al.*, 1999). There are differences between species in the expression and localization of cholecystokinin receptors that may influence the possible production of pancreatic tumors (Bourassa *et al.*, 1999; Holicky *et al.*, 2001).

The study reported here tested the hypothesis that the male cynomolgus monkey, as a representative primate, does not respond to the effects on the liver, pancreas, and testes that have been associated with APFO and other peroxisome proliferating compounds in the male rat. The specific aims of this study, in addition to observing descriptive toxicity endpoints, were to assess the effect of chronic (26-week) APFO treatment on biological markers associated with the hepatic, pancreatic, and testicular responses seen in the rat with APFO and other peroxisome proliferating compounds. These biological markers included measurement of acyl CoA oxidase activity, replicative DNA synthesis, hormone levels including estradiol and CCK, as well as indications of cholestasis including bilirubin, alkaline phosphatase, and bile acid determination. A three-month recovery phase was added to observe for delayed effects and reversibility of effects. The design of this study, which also included assessment of PFOA concentrations in serum and liver by modern methods of analysis, incorporated significant enhancements over the monkey rhesus study (Griffith and Long, 1980).

MATERIALS AND METHODS

Animals and husbandry. Male cynomolgus monkeys were obtained from Covance Research Products Inc. (Denver, PA) in August 1998. The mean age of the monkeys, as estimated by dentition, was 6 ± 1 (SD) years and ranged from 3 to 9 years. The monkeys weighed 3.2 to 4.5 kg at initiation of treatment. Monkeys were acclimated for 35 days at Covance Laboratories in Madison, Wisconsin before initiation of treatment. Each monkey was assigned a permanent number upon arrival and identified with a collar tag. The monkeys were housed individually in suspended stainless-steel cages. The animal room was environmentally controlled to maintain 18 to 29°C, a relative humidity of 30 to 70%, and a 12-h light/dark cycle.

Certified primate diet (#8726C, Harlan Teklad) was provided once or twice daily along with a variety of fresh and dried fruits as well as various breakfast cereals. Water was provided *ad libitum*. Samples of the water were analyzed for specific microorganisms and contaminants. During the study, several monkeys experiencing low food consumption in the high-dose group were offered supplements to rehydrate the monkeys and stimulate food consumption.

Monkeys were assigned to treatment groups using a computerized blocking

procedure designed to achieve body weight balance within each treatment group.

Materials, dose preparation, and treatment. Ammonium perfluorooctanoate (APFO, lot 332) was provided by 3M (St. Paul, MN; purity 95.2%). Dose levels described here have not been adjusted for purity. Impurities that could contribute to toxicity included perfluorohexanoate (0.73%) and perfluoroheptanoate (3.76%). Gelatin capsules (Torpac, Inc., Fairfield, NJ, Size No. 2, Lot No. 122932) containing the appropriate dose of APFO were used for dose administration. Control monkeys received empty gelatin capsules. Capsules were prepared at least weekly. Individual daily doses were calculated based on the most recently recorded body weights. The dose preparations were stored at room temperature. Since the APFO was added directly to capsules as supplied without the use of a vehicle, no dose analysis was necessary.

Dose levels were 0 (control group, $n = 6$), 3 mg/kg (low-dose group, $n = 4$), 10 mg/kg (mid-dose group, $n = 6$), and 30, later reduced to 20 (30/20) mg/kg on Day 22 (high-dose group, $n = 6$). The capsules were administered orally once a day, 7 days per week, for at least 26 weeks (182 days) except as noted below. One monkey in the low-dose group needed to be replaced for nontreatment-related issues. The replacement monkey (Monkey 5721) received his initial dose on Day 17. High-dose monkeys were initiated on 30 mg/kg; however, due to toxicity, dosing was discontinued from day 12 through 21 and recommenced on Day 22 at 20 mg/kg. Also due to toxicity, dose administration was discontinued for three high-dose monkeys on Day 43 (Monkey 5711), Day 66 (Monkey 5722), and Day 81 (Monkey 5703). Two monkeys in each of the control and mid-dose groups were designated as recovery group monkeys and were observed for reversibility, persistence, or delayed occurrence of toxic effects for 13 weeks after the 26-week treatment period.

Clinical observations. The monkeys were observed twice daily for general health, behavior, and qualitative food consumption. Ophthalmic examinations were conducted on each monkey before treatment and at week 27. Recovery monkeys also were given ophthalmic exams at week 40. Body weights were recorded weekly before initiation of treatment, on the day before initiation of treatment for dose calculation, on the first day of treatment, and weekly thereafter.

Clinical pathology. A clinical laboratory evaluation was conducted before initiation of treatment, on Days 31, 63, 91, and 182 of treatment and during recovery on Days 217, 245, and 275. At each sampling time, blood and urine samples were collected from monkeys that had been fasted overnight. Urine was collected overnight on wet ice before blood sampling; water was provided *ad libitum*. Blood was collected from the femoral vein. Sodium citrate was used as the anticoagulant for coagulation tests, and potassium EDTA was the anticoagulant used for hematology tests. Blood was collected from monkeys with unscheduled sacrifices. Otherwise, monkeys with scheduled collections were bled in random order.

Hematological parameters (blood cell counts [erythrocytes, leukocytes, and platelets], hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration) were measured or calculated using an Abbott Cell-Dyn 3500 hematology analyzer, as were differential cell counts of segmented neutrophils, lymphocytes, eosinophils, and basophils. Blood cell morphology and reticulocyte counts were obtained using a Miller Disk microscopy technique. Bone marrow smears were prepared at euthanization. Coagulation parameters measured were prothrombin time, activated partial thromboplastin time (nephelometry), and fibrinogen.

Clinical chemical analytes were measured on a Hitachi® Model 704,911 Chemistry Analyzer using the Roche Diagnostics, Hitachi® 704,911 Operator Reference Manual, Method Application. These clinical chemistry measurements included glucose (enzymatic/glucose-hexokinase), urea nitrogen (kinetic enzymatic urease/GLDH), creatinine (kinetic—modified Jaffe), total protein (biuret), albumin (bromocresol green), globulin (calculated—total protein minus albumin), total bilirubin (photometric determination of azobilirubin complex after coupling with diazotized sulfanilic acid), cholesterol (kinetic enzymatic

without extraction using Trinder reaction), triglycerides (kinetic enzymatic without blank), aspartate aminotransferase (kinetic—NADH consumption with oxaloacetate), alanine aminotransferase (kinetic—NADH consumption with pyruvate), alkaline phosphatase (kinetic using p-nitrophenyl-phosphate), gamma-glutamyl transferase (kinetic using glutamyl-p-nitroanilide), sorbitol dehydrogenase (D-fructose NADH UV), creatine kinase (kinetic using N-acetyl cysteine), calcium (cresolphthalein complexing), inorganic phosphorus (UV—phosphomolybdate), sodium (ion-selective electrode—indirect), potassium (ion-selective electrode—indirect), chloride (ion-selective electrode—indirect), total bile acids (enzymatic), amylase (enzymatic), lipase (enzymatic), and pancreatic-specific amylase (enzymatic).

Urine was analyzed for color, clarity, volume (approximately 16 h overnight), specific gravity, pH, protein, glucose, ketones, bilirubin, blood, urobilinogen, microscopic examination of sediment, and general appearance. Color and clarity were determined by observation. Volume was determined to the nearest ml in a graduated cylinder. Specific gravity was determined on an AOTS refractometer (temperature compensated) using one large drop of urine. Measurement of pH, protein, glucose, ketones, bilirubin, blood, and urobilinogen was accomplished colorimetrically with Multistix® strips. After color, clarity, volume, specific gravity, and Multistix determinations, the urine was centrifuged, the supernatant poured off, and a drop of sediment placed on a glass slide. The slide was scanned under low power light microscopy for crystals and casts and then under high power to count other sediment constituents.

Determination of hormones. Blood was collected from the femoral vein of nonfasted monkeys 18, 8, and 4 days prior to initiation of treatment (Study Days -18, -8, and -4) and on Days 35, 66, 94, and 183 of treatment. Blood collection from recovery monkeys occurred on Study Days 220, 248, and 276 after initiation of treatment. Blood samples were split into serum and plasma samples for further analysis for various hormones. Plasma samples were analyzed for cholecystokinin and testosterone (with the exception of recovery monkeys in which case serum samples were used for testosterone). Serum was analyzed for estradiol, estrone, estriol, thyroid stimulating hormone, total and free triiodothyronine, and total and free thyroxine.

Serum levels of cortisol, testosterone, estradiol, estrone, estriol, free and total triiodothyronine (FT₃ and TT₃) and free and total thyroxine (FT₄ and TT₄) were measured at Anilytics, Inc., Gaithersburg, MD, using radioimmunoassay methods under Anilytics, Inc. standard operating procedures. ICN Pharmaceuticals radioimmunoassay (RIA) kits were used for determination of cortisol, testosterone, estradiol, and estrone. Total estriol was measured using a Coat-A-Count Total Estriol kit (Diagnostic Products Corporation, Los Angeles, CA). Thyroid stimulating hormone (TSH) was analyzed by an in-house method at Anilytics, Inc. (Gaithersburg, MD) employing a double-antibody RIA procedure using human reagents (Anilytics, Inc. Standard Operating Procedure for Non-Human Primate Thyroid Stimulating Hormone [TSH], May 12, 1998). FT₃, TT₃, FT₄, and TT₄ were determined using Coat-A-Count RIA kits (Diagnostic Products Corporation, Los Angeles, CA).

Frozen plasma samples were sent to Haskell Laboratory for CCK analysis. Plasma samples were extracted over a C-18 Sep Pak column (Supelco Co., Bellefonte, PA) washed with 0.05% trifluoroacetic acid (TFA), and eluted with 50% acetonitrile/50% TFA. Extracted samples were dried under vacuum and reconstituted prior to analysis by RIA (Obourm *et al.*, 1997).

Liver biochemistry. Samples of the right lateral lobe of the livers from each monkey, scheduled and unscheduled sacrifices, were flash-frozen in liquid nitrogen at necropsy and analyzed for palmitoyl CoA oxidase activity by the method of Bronfman *et al.* (1979).

Bile (up to 5 ml) was collected at sacrifice of each monkey, flash frozen in liquid nitrogen and analyzed for specific bile acids. Bile samples were stored frozen (-70°C) prior to analysis. After thawing, bile acids were extracted from a 20 µl aliquot of bile using solid phase extraction on Isoelute C18 cartridges. The extracted samples were reconstituted in HPLC grade methanol, filtered through 0.45µ nylon filter, and subjected to HPLC analysis on a C18 reversed phase column (25 cm, Apex ODS 5 m) using a 75% (v/v) methanol/ 25% (v/v) potassium phosphate buffer (10 mM, pH 5.44) mobile phase. Bile acids

(taurocholic acid, glycocholic acid, taurochenodeoxycholic acid, taurodeoxycholic acid, glycochenodeoxycholic acid, glycodeoxycholic acid, tauroolithocholic acid, and glycolithocholic acid) were quantified against authentic standards by UV detection at 200 nm using Chromeleon software.

Determination of serum and liver PFOA content. Approximately 2 ml of whole blood (for serum) were collected from the femoral vein of nonfasted monkeys during Study Week 2 and every two weeks thereafter.

A section of liver was collected at sacrifice from each monkey, weighed, flash frozen in liquid nitrogen, and stored at -60 to -80°C until shipped on dry ice to 3M for analysis.

PFOA levels in sera and liver were determined using an ion-pairing extraction followed by quantitative tandem mass spectrometry; the method is described in detail elsewhere (Hansen *et al.*, 2001). Briefly, the serum or liver sample was thoroughly mixed with a buffered solution of tetra-butyl ammonium hydrogen sulfate. The PFOA ion pair was partitioned into methyl *tert*-butyl ether, an exact volume of which was removed from the solution, concentrated under nitrogen, and reconstituted in methanol. Liver samples were homogenized in water prior to extraction.

The extracts were analyzed by high performance liquid chromatography electrospray tandem mass spectrometry in the negative ion multiple response monitoring mode. A characteristic product ion produced by fragmentation of the primary ion was monitored quantitatively and evaluated versus an extracted curve weighted $1/x$.

Concurrent with blood collections for PFOA determination, at least 2 ml of urine was collected on wet ice and at least 5 g of feces (overnight) were collected from each monkey. Results of urine and fecal analysis will be discussed in a future paper.

Anatomic pathology. After 26 weeks of treatment, 4 monkeys in the control group, the three surviving monkeys in the 3 mg/kg dose group, 4 monkeys in the 10 mg/kg dose group, and the 5 surviving monkeys in the 30/20 mg/kg dose group were fasted overnight, anesthetized with ketamine and xylazine, weighed, exsanguinated, and necropsied. Similarly, the two monkeys in the control and 10 mg/kg dose recovery groups were sacrificed 13 weeks following the last dose on Day 182. Necropsies were also performed on the 3 mg/kg and 30/20 mg/kg dose-group monkeys that had unscheduled sacrifices on treatment Days 137 and 29, respectively. The adrenals, brain, epididymis, kidneys, liver, pancreas, testes, and thyroids with parathyroids were collected and weighed. Organ to body weight and organ to brain weight ratios were calculated.

The following were collected for microscopic evaluation: adrenal, aorta, brain, cecum, colon, duodenum, epididymis, esophagus, eyes, femur, gall bladder, heart, ileum, jejunum, kidney, lesions, liver, lung, mammary gland, mesenteric lymph node, pancreas, pituitary, prostate, rectum, salivary gland (mandibular), sciatic nerve, seminal vesicles, skeletal muscle (thigh), skin, spinal cord, spleen, sternum with bone marrow, stomach, testes, thyroid, trachea, and, urinary bladder. Tissues were embedded in paraffin, stained with hematoxylin and eosin, and examined by light microscopy.

Replicative DNA synthesis. Representative samples of the left lateral lobe of the liver, left and right testes and pancreas were collected from each monkey. After fixation (formalin or zinc formalin), samples were embedded in paraffin, and sections ($\sim 5\ \mu\text{m}$) were prepared. Previously described methods were used to stain tissues for PCNA (Eldridge *et al.*, 1993). Briefly, tissue sections were incubated with a monoclonal antibody to PCNA (Lot # 107 DAKO, Carpinteria, CA) and reagents required for the avidin-biotin peroxidase method (MslgG Kit Lot # PK6102 Vector, Burlingame, CA) for the detection of the antigen-antibody complex. PCNA expression in cells in all stages of the cell cycle was localized by the chromagen, 3,3'-diaminobenzidine (DAB Lot # 108H8210, Sigma Chemical Co., St. Louis, MO). Tissue sections were counterstained with hematoxylin. Liver cell proliferation was determined by scoring the percent of cells in S-phase among at least 3000 cells in ten randomly selected fields per monkey. For the pancreas, islet and exocrine cells on an entire tissue section were observed and scored subjectively with "3" representing cases in which exocrine cells stained greater than islets and "4" representing cases in which islets and exocrine cells were heavily stained. In

the case of the testes, the tissue section furthest from the slide label was scored and the percentage of Leydig cells in S-phase was determined by scoring at least 100 cells on each section.

Statistics. Differences from control values were determined by one-way ANOVA, if applicable, and where significant, followed by Dunnett's test used for control versus treated group comparisons. Paired *t*-tests were used to compare each treatment mean to its baseline value. One-way analysis of covariance (ANCOVA) was used to analyze body weights with initial body weight as the covariate. *p*-values less than 0.05 were considered to be statistically significant. The highest biological significance was attributed when both the within-dose group and time-related pair-wise comparisons were both statistically significant. For interpretive purposes, palmitoyl CoA oxidase activities less than 2-fold greater than control activities were not considered to represent an adverse effect and were considered to reflect natural variation. Only data collected on or after the first day of treatment were analyzed statistically. Data collected before the first day of treatment or during recovery (except for hormone analyses data) were not analyzed statistically.

RESULTS

Animals and Husbandry

One 3 mg/kg/day dose-group monkey was sacrificed on Day 14 due to bacterial septicemia, a condition not believed to be related to treatment. This monkey was replaced on Day 15. The replacement monkey began dosing on Day 17.

Clinical Observations

Overall, body-weight changes as a percent of initial prestudy body weight in the 30/20 mg/kg dose group were significantly lower than controls over the 27 week period (Fig. 1). During the first week of dosing, all monkeys in the 30 mg/kg/day dose group had qualitatively low food consumption and lost from 3.1 to 7.5% of body weight. Four of the 6 monkeys also had few or no feces. Dosing was suspended on Day 12. On Day 22, dosing was reinitiated at a lower dose, 20 mg/kg/day. After commencing the 20 mg/kg/day dosing on Day 22, weight change as a percent of prestudy weight continued to be significantly depressed compared to the other groups (Fig. 1). Two monkeys in this group remained on this regimen for the intended 6-month dosing period. By the end of the dosing period, these two monkeys showed a mean gain in body weight of 5% (individually, -8 and 18%) over their predosing weight, compared to 19, 20, and 20% for the control, 3 mg/kg, and 10 mg/kg groups, respectively (Fig. 1). Dosing of three monkeys in this group was discontinued on Days 43, 66, or 81, respectively. These three monkeys exhibited low or no food consumption, few or no feces, and dramatic body-weight losses (18 – 23%) prior to being suspended permanently from dosing (Fig. 2). These three monkeys regained their lost weight over the remainder of the study and experienced gains over their predosing weights of 5, 16, and 29% (Fig. 2). The sixth monkey in this group was sacrificed in moribund condition on Day 29. In addition to the signs noted above for the other monkeys in this group, this monkey had lost 12.5% of body weight, was notably hypoactive, and cold to the touch.

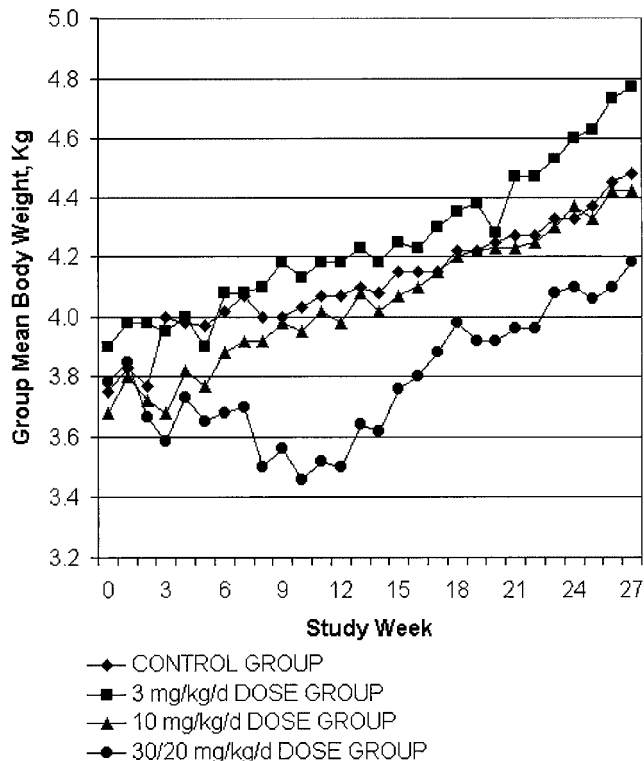


FIG. 1. Group mean body weight for male cynomolgus monkeys through 26 weeks of daily po capsule dosing with ammonium perfluorooctanoate. Monkeys in the 30/20 mg/kg/day dose group experienced weight loss, in the first weeks of the study after dosing with 30 mg/kg/day, and this toxicity continued for 4 of the 6 monkeys in this dose group after reducing the dose to 20 mg/kg/day (see Fig. 2).

Clinical signs, food consumption, and body weights were normal in all monkeys receiving 3 and 10 mg/kg with the exception of one anomalous occurrence in a 3 mg/kg monkey. This low-dose monkey was sacrificed in moribund condition on Day 137. In addition to few feces, low food consumption, and loss of approximately 10% body weight in 1 week, this monkey exhibited hind-limb paralysis, ataxia, and lack of response to a painful stimulus in the week prior to sacrifice. No ophthalmic effects in any of the APFO-treated monkeys were noted.

Two control and two 10 mg/kg dose-group monkeys were observed for delayed effects for 13 weeks after the end of the 26-week dosing period. The 10 mg/kg dose-group recovery monkeys experienced body-weight changes of 5 and -3% of Week 27 (end of dosing period) weights as compared to the control recovery monkeys, who experienced weight gains of 10 and 11% of their Week 27 weights (Fig. 3).

Clinical Pathology

With the exception of individual observations and small but significant increases in triglycerides noted on Days 31, 63, and

91 in the 30/20 mg/kg/day dose group, no major findings were present in the clinical chemistry, hematology, and urinalysis that could be attributed to treatment. The group mean data for glucose, cholesterol, triglycerides, alkaline phosphatase, alanine transaminase, and total bilirubin are presented in Table 1.

Administration of APFO at 3 or 10 mg/kg produced no apparent effects on measured clinical parameters including hematology, coagulation, clinical chemistry, and urinalysis. However, the 3 mg/kg/day dose group monkey that was sacrificed in moribund condition on Day 137 showed marked hyperfibrinogenemia, moderate lymphopenia, moderate hypoalbuminemia, and mild hypocholesterolemia. It should be noted that 11 days prior to initiation of treatment (Day -11), this monkey had a high hematocrit (49.7%) and albumin concentration (5.6 g/dl), possibly indicative of mild dehydration, and it had the lowest neutrophil count (1600/ μ l) of all monkeys.

Findings in the 30/20 mg/kg/day dose group were compli-

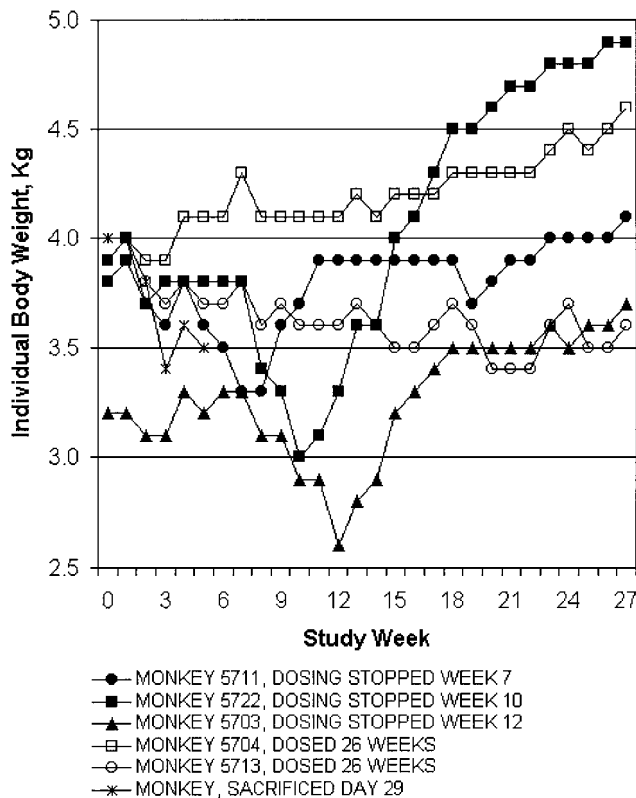


FIG. 2. Individual body weight for male cynomolgus monkeys during daily po capsule dosing with 30/20 mg/kg/day ammonium perfluorooctanoate. Monkeys in the 30/20 mg/kg/day dose group experienced toxicity, including weight loss, in the first weeks of the study after dosing with 30 mg/kg/day. Dosing was suspended during study week 2 and started again at 20 mg/kg/day at the beginning of study week 4. One monkey in this group was sacrificed in moribund condition at the beginning of week 5, and dosing was stopped due to excessive toxicity, including weight loss, for three other monkeys in the 30/20 mg/kg/day dose group in weeks 7, 10, and 12, respectively. The latter three monkeys regained body weight after dosing was stopped. Only two monkeys in the 30/20 mg/kg/day dose group were treated through study week 26.

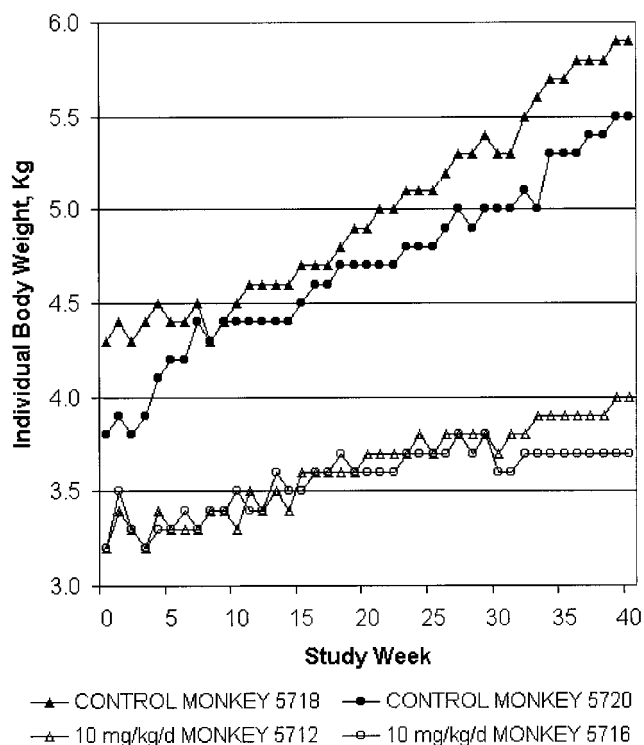


FIG. 3. Individual body weight for control and 10 mg/kg/day dose group male cynomolgus monkeys during 26 weeks of po capsule dosing and 14 weeks of posttreatment recovery.

cated by the unscheduled sacrifice of one monkey on Day 29 and the cessation of treatment for three others by Day 81. Only two monkeys remained on treatment during clinical pathology testing intervals on Days 91 and 182. In this small group, mild increases in triglycerides and mild to marked decreases in absolute neutrophil count, total protein concentration, and albumin concentration occurred. The differences in triglycerides were statistically significant on Days 31 and 182 versus pretreatment values. The other differences were not statistically significant but were consistent over time. Absolute neutrophil counts and albumin concentrations were mildly decreased prior to cessation of treatment for the three monkeys for which dosing was suspended. On Day 63, the 30/20 mg/kg dose-group monkey for which daily dosing was stopped on Day 66 had approximately 10-fold increases over the means in serum activities of aspartate aminotransferase (419 IU/l), alanine aminotransferase (595 IU/l), and creatine kinase (1122 IU/l), as well as a 5-fold increase in sorbitol dehydrogenase (20 IU/l) and an increase in serum bile acids. The monkey that was sacrificed in moribund condition on Day 29 had markedly elevated serum activities of aspartate aminotransferase (1974 IU/l), alanine aminotransferase (1463 IU/l), sorbitol dehydrogenase (59 IU/l), and creatine kinase (68,850 IU/l) as well as markedly low cholesterol (14 mg/dl).

Hormone Determinations

Data for thyroid hormones, estradiol, testosterone, and cholecystokinin are presented in Table 2. No effects on estrone, estriol, or testosterone were noted. Mean group estradiol values in all groups including the controls tended to be considerably lower during the treatment period than the corresponding pretreatment values. These values appeared to be lower in the high-dose group monkeys than in the other groups. The estradiol values in the recovery monkeys tended to be similar to the predose values. No significant changes in testosterone values were seen during the treatment period. In the last recovery sampling, mean values (9 and 15 ng/ml) tended to be higher than means during the rest of the study (1–8 ng/ml).

TSH and free and total thyroxine (FT₄ and TT₄) were not altered in a significant manner during the study. In all groups, FT₃ and TT₃ values taken on predosing Days –18 and –8 (data not shown) were considerably higher than those obtained on predosing Day –4 and throughout the remainder of the dosing period; therefore, the Day –4 values were considered the most appropriate prestudy values for comparison. With free and total triiodothyronine (FT₃ and TT₃), mean group values from predosing Day –4 to the end of the study remained relatively constant; although, monkeys in the 30/20 mg/kg dose group exhibited generally lower group mean values. The mean individual changes in FT₃ and TT₃ over the period that each monkey was being dosed were decreased compared to controls in a statistically significant manner in the 30/20 mg/kg/day dose group. For the three 30/20 mg/kg/day dose-group monkeys suspended from dosing, FT₃ and TT₃ tended to increase toward prestudy values after suspension of dosing.

No biologically significant alterations in relation to dose groups in plasma CCK concentrations were observed.

Liver Biochemistry

Hepatic DNA content and enzymes that are specific markers for different subcellular fractions were determined in an attempt to explain the increased liver weights observed in these studies. APFO administration resulted in a small increase in peroxisomal CN⁻-insensitive palmitoyl CoA oxidation and a marked increase in mitochondrial succinate dehydrogenase activity. No biologically significant effects were seen on acid phosphatase or glucose-6-phosphatase activities; these are markers for lysosomes and endoplasmic reticulum respectively (Table 3). Additionally, there appeared to be a dose-related decrease in hepatic DNA concentration. There were no significant differences between control and treated groups with respect to the amount and distribution of bile acids (Table 4).

PFOA Concentration in Serum and Liver

Although considerable variability in measurement occurred, steady-state values for PFOA in the serum appeared to have been attained with 4 to 6 weeks of daily dosing. Serum con-

TABLE 1
Clinical Chemistries in Male Cynomolgus Monkeys Dosed Orally for 6 Months with Ammonium Perfluorooctanoate

| | Treatment week (day) | | | | |
|-----------------------------|----------------------|---------------------|---------------------|----------------------|----------------------|
| | -2 (-11) | 5 (31) | 10 (63) | 14 (91) | 27 (182) |
| Glucose (mg/dl) | | | | | |
| Control | 93 ± 15 | 78 ± 28 | 78 ± 21 | 79 ± 12 | 71 ± 14 |
| 3 mg/kg/day | 88 ± 12 ^a | 78 ± 9 | 68 ± 7 | 78 ± 10 | 63 ± 8 ^b |
| 10 mg/kg/day | 92 ± 22 | 73 ± 9 | 71 ± 11 | 77 ± 9 | 66 ± 20 |
| 30/20 mg/kg/day | 81 ± 5 | 75 ± 9 ^c | 76 ± 7 ^d | 85 ± 19 ^e | 81 ± 28 ^e |
| Cholesterol (mg/dl) | | | | | |
| Control | 147 ± 44 | 146 ± 19 | 151 ± 37 | 167 ± 45* | 156 ± 35 |
| 3 mg/kg/day | 174 ± 54 | 151 ± 60 | 161 ± 61 | 157 ± 51 | 142 ± 47 |
| 10 mg/kg/day | 145 ± 22 | 142 ± 26 | 158 ± 46 | 155 ± 31 | 154 ± 31 |
| 30/20 mg/kg/day | 140 ± 26 | 158 ± 21 | 146 ± 12 | 142 ± 9* | 150 ± 16 |
| Triglycerides (mg/dl) | | | | | |
| Control | 39 ± 11 | 44 ± 23 | 43 ± 14 | 40 ± 9 | 44 ± 9 |
| 3 mg/kg/day | 50 ± 10 | 51 ± 24 | 59 ± 20 | 56 ± 25 | 51 ± 24 |
| 10 mg/kg/day | 61 ± 18** | 76 ± 27* | 76 ± 27* | 88 ± 37*** | 72 ± 25 |
| 30/20 mg/kg/day | 57 ± 13 | 108 ± 57*** | 67 ± 39 | 99 ± 55 | 92 ± 40** |
| Alkaline phosphatase (IU/l) | | | | | |
| Control | 455 ± 202 | 577 ± 232* | 582 ± 193* | 529 ± 240 | 444 ± 191 |
| 3 mg/kg/day | 556 ± 180 | 629 ± 194 | 612 ± 170* | 585 ± 178 | 574 ± 290 |
| 10 mg/kg/day | 604 ± 174 | 704 ± 165* | 668 ± 190 | 656 ± 209 | 544 ± 181 |
| 30/20 mg/kg/day | 521 ± 232 | 551 ± 285 | 484 ± 160 | 432 ± 306 | 384 ± 261 |
| Alanine transaminase (IU/l) | | | | | |
| Control | 58 ± 29 | 49 ± 17 | 62 ± 24 | 63 ± 23 | 68 ± 16 |
| 3 mg/kg/day | 42 ± 8 | 29 ± 6 | 45 ± 27 | 47 ± 25 | 43 ± 6 |
| 10 mg/kg/day | 77 ± 53 | 55 ± 23 | 53 ± 19 | 53 ± 16 | 53 ± 27 |
| 30/20 mg/kg/day | 67 ± 42 | 36 ± 14 | 192 ± 270 | 48 ± 18 | 49 ± 8 |
| Total bilirubin (mg/dl) | | | | | |
| Control | 0.0 ± 0.1 | 0.2 ± 0.1 | 0.5 ± 0.2* | 0.2 ± 0.2* | 0.3 ± 0.1* |
| 3 mg/kg/day | 0.1 ± 0.2 | 0.1 ± 0.1 | 0.3 ± 0.1* | 0.3 ± 0.1* | 0.2 ± 0.2 |
| 10 mg/kg/day | 0.2 ± 0.2 | 0.2 ± 0.1 | 0.2 ± 0.1** | 0.2 ± 0.1 | 0.3 ± 0.2 |
| 30/20 mg/kg/day | 0.2 ± 0.2 | 0.1 ± 0.1 | 0.3 ± 0.2 | 0.1 ± 0.1 | 0.3 ± 0.4 |

Note. Group means ± SD. Statistics reflect only those animals receiving treatment when blood was drawn, including comparisons to prestudy values. Control group and 10 mg/kg/day group, $n = 6$; 3 mg/kg/day group, $n = 4$, unless otherwise noted; 30/20 mg/kg/day group, $n = 6$, unless otherwise noted.

^aMonkey 5721 was added to study in Week 3 (Day 17) to replace a monkey (5723).

^b $n = 3$. Monkey 5721 was sacrificed in moribund condition in Week 20 (Day 137).

^c $n = 5$. Monkey 5724 was sacrificed in moribund condition in Week 5 (Day 29).

^d $n = 4$. Dosing of monkey 5711 was suspended in Week 7 (Day 43).

^e $n = 2$. Dosing of monkeys 5722 and 5703 was suspended in Weeks 10 (Day 66) and 12 (Day 81), respectively.

*Significantly different from pretreatment values (week 2) by a two-tailed, paired Student's t -test ($p < 0.05$)

**Significantly different from time-related control using Dunnett's t -test ($p < 0.05$, 2 tailed).

centration of PFOA did not increase in a linear dose-dependent manner. Assuming that measurements made at or after Week 6 represent repeat measures at steady state, mean serum concentrations ± SD (range of values, number of samples) for the 3 mg/kg, 10 mg/kg, and 30/20 mg/kg dose groups were 77 ± 39 μg/ml (10–154, 44), 86 ± 33 μg/ml (10–180, 70), and 158 ± 100 μg/ml (20–467, 33) μg PFOA/ml, respectively. Two-thirds (43/64) of the control monkey serums in this time period had detectable PFOA above the limit of quantitation of 0.014 μg/ml, and these averaged 0.203 ± 0.154 μg/ml. The mean values for the 3 mg/kg and 10 mg/kg dose groups were not significantly different at the $p = 0.05$ level. However, the mean for the 30/20 mg/kg/day dose group was significantly different

from both lower dose groups ($p < 0.001$). In the recovery group monkeys (10 mg/kg), serum PFOA concentrations returned to baseline by the end of the 90-day recovery period.

Liver tissue PFOA concentrations measured in the monkeys are represented in Table 5. As with serum, the PFOA concentrations in liver did not increase in linear proportion to dose. The values at terminal sacrifice just after dosing for the 3 mg/kg and 10 mg/kg dose groups were similar, and ranged from 6.29 to 21.9 μg PFOA/g tissue. The 30/20 mg/kg/day dose-group values for the two monkeys sacrificed on Day 183 were 16.0 and 83.3 μg PFOA/g tissue. It is noteworthy that the highest liver tissue value obtained (154 μg PFOA/g tissue) was from the monkey sacrificed *in extremis* on Day 29. Liver tissue

TABLE 2
Hormones in Male Cynomolgus Monkeys Dosed Orally for 6 Months with Ammonium Perfluorooctanoate

| | Treatment week (day) | | | | |
|-------------------------------------|--------------------------|--------------------------|--------------------------|--------------------------|----------------------------|
| | -1 (-4) | 5 (35) | 10 (66) | 14 (94) | 27 (183) |
| Thyroid stimulating hormone (mU/ml) | | | | | |
| Control | 0.37 ± 0.36 | 0.23 ± 0.23 | 0.23 ± 0.34 | 0.54 ± 0.46 | 0.40 ± 0.23 |
| 3 mg/kg/day | 0.53 ± 0.58 ^a | 0.36 ± 0.53 | 0.19 ± 0.27 | 0.60 ± 0.55 | 0.65 ± 0.17 ^{b,*} |
| 10 mg/kg/day | 0.46 ± 0.57 | 0.26 ± 0.44 | 0.56 ± 1.07 | 0.38 ± 0.53 | 0.87 ± 1.09* |
| 30/20 mg/kg/day | 0.29 ± 0.28 | 0.03 ± 0.04 ^c | 0.22 ± 0.25 ^d | 0.20 ± 0.11 ^e | 0.39 ± 0.10 ^e |
| Total thyroxine (μg/dl) | | | | | |
| Control | 5.13 ± 0.81 | 4.72 ± 0.95 | 4.65 ± 0.86 | 4.22 ± 0.90 | 3.84 ± 0.77 |
| 3 mg/kg/day | 5.16 ± 1.28 | 4.02 ± 0.76 | 3.52 ± 0.53 | 3.55 ± 0.38 | 2.58 ± 0.17** |
| 10 mg/kg/day | 4.17 ± 0.51 | 2.95 ± 0.46** | 3.04 ± 0.46** | 2.93 ± 0.42** | 2.71 ± 0.35** |
| 30/20 mg/kg/day | 4.51 ± 0.79 | 3.70 ± 0.29 | 3.27 ± 1.02** | 3.76 ± 0.42 | 2.61 ± 0.25** |
| Free thyroxine (μg/dl) | | | | | |
| Control | 1.77 ± 0.29 | 1.60 ± 0.34 | 1.48 ± 0.26 | 1.49 ± 0.50 | 1.55 ± 0.43 |
| 3 mg/kg/day | 1.72 ± 0.35 | 1.47 ± 0.16 | 1.34 ± 0.30 | 1.32 ± 0.28 | 1.04 ± 0.04 |
| 10 mg/kg/day | 1.56 ± 0.18 | 1.09 ± 0.17** | 1.08 ± 0.20** | 1.06 ± 0.20 | 0.96 ± 0.13** |
| 30/20 mg/kg/day | 1.56 ± 0.26 | 1.23 ± 0.06** | 1.08 ± 0.26 | 1.34 ± 0.27 | 0.90 ± 0.24** |
| Total triiodothyronine (ng/dl) | | | | | |
| Control | 155 ± 18 | 171 ± 16 | 163 ± 19 | 162 ± 25 | 157 ± 15 |
| 3 mg/kg/day | 150 ± 26 | 168 ± 30 | 170 ± 21* | 177 ± 28* | 134 ± 17 |
| 10 mg/kg/day | 170 ± 23 | 152 ± 15 | 162 ± 10 | 157 ± 18 | 135 ± 23 |
| 30/20 mg/kg/day | 148 ± 16 | 110 ± 39** | 90 ± 51** | 120 ± 35 | 104 ± 33** |
| Free triiodothyronine (ng/dl) | | | | | |
| Control | 6.39 ± 0.63 | 5.49 ± 0.70 | 5.71 ± 1.14 | 5.02 ± 0.73 | 5.62 ± 0.89 |
| 3 mg/kg/day | 6.06 ± 0.63 | 5.58 ± 0.65 | 6.23 ± 0.59 | 5.28 ± 0.34 | 4.87 ± 0.12 |
| 10 mg/kg/day | 6.16 ± 0.44 | 5.13 ± 0.41 | 5.31 ± 0.54 | 4.98 ± 0.89 | 4.67 ± 0.64 |
| 30/20 mg/kg/day | 6.00 ± 0.88 | 3.78 ± 1.18** | 3.01 ± 1.96** | 4.46 ± 0.76 | 3.39 ± 1.54** |
| Testosterone (ng/ml) | | | | | |
| Control | 3.76 ± 3.46 | 2.22 ± 2.63 | 4.76 ± 3.45 | 4.63 ± 4.31 | 7.49 ± 4.62 |
| 3 mg/kg/day | 6.67 ± 6.55 | 3.03 ± 2.99 | 3.68 ± 2.26 | 7.36 ± 2.66 | 7.81 ± 4.27 |
| 10 mg/kg/day | 2.47 ± 2.42 | 2.00 ± 2.02 | 5.15 ± 3.92 | 2.89 ± 2.06 | 7.83 ± 3.69* |
| 30/20 mg/kg/day | 3.97 ± 3.21 | 0.81 ± 0.49 | 2.76 ± 3.01 | 1.25 ± 0.09 | 1.74 ± 0.44 |
| Estradiol (pg/ml) | | | | | |
| Control | 24.9 ± 6.3 | 7.6 ± 7.0 | 15.0 ± 12.5 | 13.5 ± 10.1 | 10.8 ± 17.0 |
| 3 mg/kg/day | 35.1 ± 9.7 | 14.7 ± 12.7 | 18.3 ± 11.5 | 7.7 ± 7.8 | 13.6 ± 11.1 |
| 10 mg/kg/day | 30.2 ± 7.4 | 11.2 ± 7.4 | 19.8 ± 10.1 | 6.4 ± 7.9 | 7.8 ± 6.2 |
| 30/20 mg/kg/day | 27.7 ± 6.7 | 4.6 ± 7.1 | 2.1 ± 4.2 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Cholecystokinin (Fmol/mR) | | | | | |
| Control | 1.76 ± 0.55 | 3.15 ± 0.86* | 3.07 ± 1.52* | 1.79 ± 0.59 | 2.43 ± 0.84* |
| 3 mg/kg/day | 1.81 ± 0.82 | 3.02 ± 1.28* | 3.90 ± 2.69 | 1.38 ± 0.73 ^f | 3.03 ± 1.42 |
| 10 mg/kg/day | 1.53 ± 0.62 | 3.10 ± 1.18* | 2.74 ± 1.30 | 1.43 ± 0.23* | 2.31 ± 0.88 |
| 30/20 mg/kg/day | 1.88 ± 1.07 | 2.55 ± 0.39 | 2.44 ± 1.10 | 1.90 ± 0.14 | 1.80 ± 1.16 |

Note. Group means ± SD. Statistics reflect only those animals receiving treatment when blood was drawn, including comparisons to prestudy values. Control group, *n* = 6; 3 mg/kg/day group, *n* = 4, unless otherwise noted; 10 mg/kg/day and 30/20 mg/kg/day groups, *n* = 6, unless otherwise noted.

^aMonkey 5721 was added to study in Week 3 (Day 17) to replace a monkey (5723).

^b*n* = 3. Monkey 5721 was sacrificed in moribund condition in Week 20 (Day 137).

^c*n* = 5. Monkey 5724 was sacrificed in moribund condition in Week 5 (Day 29).

^d*n* = 4. Dosing of monkey 5711 was suspended in Week 7 (Day 43).

^e*n* = 2. Dosing of monkeys 5722 and 5703 was suspended in Weeks 10 (Day 66) and 12 (Day 81), respectively.

^f*n* = 3. Technical problems were encountered with extraction of sample from Monkey 5706.

^g*n* = 5. Technical problems were encountered with extraction of sample from Monkey 5710.

*Significantly different from pretreatment values (Week -1) by a two-tailed, paired Student's *t*-test (*p* < 0.05).

**Significantly different from time-related control using Dunnett's *t*-test (*p* < 0.05, 2 tailed).

PFOA concentrations from 10 mg/kg dose-group monkeys sacrificed at the end of the recovery period or from the three 30/20 dose-group monkeys that were removed from dosing by

Day 81 and sacrificed on Day 183 ranged from 0.08 to 1.41 μg PFOA/g tissue. At the recovery sacrifice of the 10 mg/kg monkeys, liver PFOA concentrations had returned to normal

TABLE 3
Subcellular Marker Enzyme Activities in Male Cynomolgus Monkeys Dosed Orally for 6 Months with Ammonium Perfluorooctanoate

| Group, mg/kg (n) | DNA | SDH | PCO | AP | G6P |
|------------------|--------------|--------------|--------------|-------------|--------------|
| 0 (4) | 1.44 ± 0.28 | 0.21 ± 0.15 | 0.53 ± 0.12 | 0.78 ± 0.10 | 12.32 ± 3.11 |
| 3 (3) | 1.23 ± 0.89 | 1.77 ± 1.59 | 0.47 ± 0.13 | 0.81 ± 0.11 | 6.02 ± 0.33* |
| 10 (4) | 1.25 ± 0.37 | 0.55 ± 0.14 | 0.90 ± 0.29 | 0.80 ± 0.14 | 10.17 ± 0.63 |
| 30/20 (2) | 1.02 ± 0.17* | 1.37 ± 0.73* | 1.36 ± 0.34* | 0.55 ± 0.10 | 8.83 ± 1.41 |
| Recovery | | | | | |
| 0 (2) | 1.69 ± 0.13 | 0.48 ± 0.43 | 0.39 ± 0.00 | 0.70 ± 0.06 | 4.59 ± 1.15 |
| 10 (2) | 1.16 ± 0.15 | 1.55 ± 1.83 | 0.46 ± 0.16 | 0.76 ± 0.10 | 7.75 ± 1.63 |

Note. Group means ± SD. DNA content given in mg DNA/g liver. SDH, Mitochondrial marker; succinate dehydrogenase, μmol cytochrome c reduced/min/g liver. PCO, peroxisomal marker; CN^- -insensitive palmitoyl CoA oxidation, $\mu\text{mol}/\text{min}/\text{g}$ liver. AP, lysosomal marker; acid phosphatase, $\mu\text{mol}/\text{min}/\text{g}$ liver. G6P, endoplasmic reticulum marker; glucose-6-phosphatase, $\mu\text{mol}/\text{min}/\text{g}$ liver.

*Significantly different from control (0 mg/kg) group, $p > 0.05$.

(0.08 and 0.15 μg PFOA/g tissue compared to control range of less than the limit of quantitation to 0.23 μg PFOA/g tissue).

PFOA was excreted in urine and feces (data not shown).

Anatomic Pathology

Increases occurred in mean absolute liver weights and mean liver-to-body weight percentages in all dose groups at terminal sacrifice after 26 weeks of dosing (Table 6). Absolute liver weight was significantly elevated ($p < 0.01$) in all treated groups relative to control. Relative liver weight increases showed a positive dose-response trend, and was significantly higher than controls in the two 30/20 mg/kg/day dose-group monkeys who were dosed through scheduled termination ($p <$

TABLE 5
Liver Tissue PFOA Concentration ($\mu\text{g}/\text{g}$) in Male Cynomolgus Monkeys Dosed Orally with Ammonium Perfluorooctanoate

| Monkey ID | PFOA in liver ($\mu\text{g}/\text{g}$) | Time liver tissue was taken |
|--|--|-----------------------------------|
| Control group | | |
| 5709 | 0.09 | End of dosing, Week 27 |
| 5714 | <LOQ | End of dosing, Week 27 |
| 5715 | 0.23 | End of dosing, Week 27 |
| 5725 | <LOQ | End of dosing, Week 27 |
| 3.0 mg/kg/day dose group | | |
| 5702 | 15.2 | End of dosing, Week 27 |
| 5706 | 18.5 | End of dosing, Week 27 |
| 5717 | 11.3 | End of dosing, Week 27 |
| 5721 | 18.3 | Unscheduled sacrifice, Day 137 |
| 10 mg/kg/day dose group | | |
| 5707 | 21.9 | End of dosing, Week 27 |
| 5708 | 6.29 | End of dosing, Week 27 |
| 5710 | 8.86 | End of dosing, Week 27 |
| 5719 | 18.8 | End of dosing, Week 27 |
| 30/20 mg/kg/day | | |
| 5704 | 16.0 | End of dosing, Week 27 |
| 5713 | 83.3 | End of dosing, Week 27 |
| Control recovery monkeys | | |
| 5718 | <LOQ | End of recovery, Week 40 |
| 5720 | <LOQ | End of recovery, Week 40 |
| 10 mg/kg/day dose group recovery monkeys | | |
| 5712 | 0.15 | End of recovery, Week 40 |
| 5716 | 0.08 | End of recovery, Week 40 |
| 30/20 mg/kg/day dose group monkeys not dosed through Week 27 | | |
| 5724 | 154 | Unscheduled sacrifice, Week 5 |
| 5711 | 0.22 | Week 27, dosing suspended Week 7 |
| 5722 | 1.41 | Week 27, dosing suspended Week 10 |
| 5703 | 1.31 | Week 27, dosing suspended Week 12 |

Note. <LOQ, less than the lower limit of quantitation of 0.019 $\mu\text{g}/\text{g}$.

TABLE 4

Bile Acids ($\mu\text{mol}/\text{ml}$) in Male Cynomolgus Monkeys Dosed Orally for 6 Months with Ammonium Perfluorooctanoate

| | Control (n = 4) | 3 mg/kg/day (n = 4) ^a | 10 mg/kg/day (n = 4) | 30/20 mg/kg/day (n = 2) |
|-------|------------------------|----------------------------------|------------------------|-------------------------|
| TC | 81 ± 41 | 59 ± 28 | 79 ± 35 | 63 ± 7 |
| GC | 37 ± 22 | 36 ± 19 | 35 ± 16 | 37 ± 30 |
| TCDC | 18 ± 1 | 13 ± 4 | 16 ± 7 | 23 ± 1 |
| TDC | 51 ± 23 | 54 ± 29 | 28 ± 9 | 59 ± 24 |
| GCDC | 4.5 ± 2.8 | 5.4 ± 4.1 | 4.3 ± 2.0 | 6.7 ± 3.4 |
| GDC | 30 ± 26 | 42 ± 35 | 11 ± 6 | 24 ± 4 |
| TLiC | 2.2 ± 1.2 | 2.2 ± 0.6 | 1.1 ± 0.4 | 2.5 ± 1.2 |
| GLiC | 1.6 ± 0.9 ^b | 2.3 ± 2.5 | 0.9 ± 0.1 ^c | 1.1 ± 0.2 |
| Total | 225 ± 67 | 214 ± 31 | 176 ± 34 | 253 ± 34 |

Note. Group means ± SD. TC, taurocholic acid; GC, glycocholic acid; TCDC, taurochenodeoxycholic acid; TDC, taurodeoxycholic acid; GCDC, glycochenodeoxycholic acid; GDC, glycodeoxycholic acid; TLiC, tauroolithocholic acid; GLiC, glycolithocholic acid. Treatment values not significantly different compared to control, using Dunnett's test ($p < 0.05$, 2 tailed).

^aIncludes Monkey 5721 who was sacrificed in moribund condition in Week 20 (Day 137).

^bn = 3.

^cn = 2.

TABLE 6

Absolute and Relative Liver Weights in Male Cynomolgus Monkeys Dosed Orally for 6 Months with Ammonium Perfluorooctanoate

| Dose group (n) | Body weight | Absolute | | Relative | |
|-----------------|-------------|--------------|-----------------|---------------|------------------|
| | | Liver weight | % Control | % Body weight | % Control |
| 0 mg/kg (4) | 3947 ± 591 | 60.2 ± 6.9 | 100 | 1.5 ± 0.1 | 100 |
| 3 mg/kg (3) | 4486 ± 30 | 81.8 ± 2.8* | 135 | 1.8 ± 0.1 | 119 |
| 10 mg/kg (4) | 4447 ± 498 | 83.2 ± 9.7* | 138 | 1.9 ± 0.1 | 122 |
| 30/20 mg/kg (2) | 3925 ± 583 | 90.4 ± 4.2* | 150 | 2.4 ± 0.5* | 157 |
| Recovery | | | | | |
| 0 mg/kg (2) | 5410 ± 240 | 90.2 ± 2.5 | 100 | 1.7 ± 0.1 | 100 |
| 10 mg/kg (2) | 3932 ± 619 | 66.0 ± 5.2 | 73 ^a | 1.7 ± 0.1 | 101 ^a |

Note. Table includes only the values from dosed-group animals that received compound through the end of the scheduled dosing period. Values are mean ± SD; weights are given in g. Statistical analysis was not performed on recovery liver weights and relative liver weights.

^aCompared to control recovery monkeys as normalized to 100%.

*Statistically significant when compared to control (Dunnett's), $p < 0.01$.

0.01). The two high-dose monkeys that were treated until the end of the dosing period had absolute and relative liver weights that were significantly elevated over the other treatment groups and control. It is noteworthy that the 3 mg/kg/day dose-group monkey sacrificed in moribund condition on Day 137 had a liver-to-body weight percentage of 2.4, which is comparable to the two 30/20 mg/kg/day monkeys dosed through scheduled termination.

All macroscopic and microscopic observations were normal at the terminal sacrifice. This includes key organs such as the liver, adrenal, spleen, pancreas, and testis.

In the 10 mg/kg/day recovery monkeys, absolute and relative organ weights were not increased, nor were there any adverse gross or histopathological findings. This information indicated recovery from hepatic effects observed in this dose group at the end of the dosing period.

Two monkeys were found in moribund condition and sacrificed at unscheduled times during the dosing period. A 30/20 mg/kg/day monkey was sacrificed on Day 29 and found to have edema and inflammation of the esophagus and stomach indicative of dosing injury. This monkey also had liver lesions, including mid-zonal and centrilobular hepatocellular degeneration and necrosis, diffuse hepatocellular vacuolation, and hepatocyte basophilia in centrilobular areas indicative of liver regeneration. Involution of the thymus, a common stress response, and degeneration and necrosis of the heart, probably agonal changes, were also observed in this monkey.

A 3 mg/kg/day monkey was found in moribund condition with hind-limb paralysis and ataxia and sacrificed on Day 137. The hind limbs of this monkey were cool on final medical examination, and ketamine injected into the thigh muscle failed to reach the systemic circulation. This could indicate compromised blood supply to the hind limbs and could also be explained by a neurogenic response without shock, as in an alpha agonist response. The microscopic and macroscopic findings did not reveal evidence of gross spinal cord injury or

impaired blood circulation. In addition, the liver of this monkey appeared to be normal; however, as noted previously, the liver-to-body weight percentage was 2.4, comparable to high-dose monkeys dosed through term.

Replicative DNA Synthesis

Cell proliferation in liver, pancreas or testes as measured by proliferating cell nuclear antigen (PCNA) was not different between control and treated groups (Table 7).

DISCUSSION

This experiment was designed to look at the toxicologic effects of APFO in a nonhuman primate model, the cynomolgus monkey, which appears to resemble humans more closely than the rodent in response to peroxisome proliferating compounds. The effects of APFO treatment noted previously in the rat that are thought to be related to the occurrence of hepatocellular, pancreatic acinar cell, and Leydig cell tumors in rats were not observed in the cynomolgus monkeys in this study. Specifically, there was no increase in peroxisome proliferation as measured by palmitoyl CoA oxidase activity. Estradiol was not increased and testosterone was not decreased. No evidence of cholestasis, as evidenced by changes in bile acids, bilirubin, and alkaline phosphatase were observed, and CCK levels did not differ among control and treated groups. The results of this study demonstrated that the liver appears to be the primary target organ in the cynomolgus monkey. Hepatomegaly in the absence of notable histologic findings was present in all dose groups. Decreases in food consumption and body weight were prominent findings in the high-dose group, consistent with effects seen in rats. The exact mechanism of the toxicity of APFO observed in the monkey remains recondite and is the subject of further investigation.

Dose-level selection for this study was based on the results

TABLE 7
Cell Proliferation as Measured by PCNA in Male Cynomolgus Monkeys Dosed Orally with Ammonium Perfluorooctanoate

| Monkey ID | Liver (%) | Pancreas (labeling index) | Testes (%) |
|-------------------|-----------|------------------------------|------------|
| Control | | | |
| 5709 | 0.025 | 3 | 22.9 |
| 5714 | 0.075 | 3 | 11.0 |
| 5715 | 0.000 | 3 | 14.9 |
| 5725 | 0.074 | 4 | 27.0 |
| 5718 ^a | 0.025 | 3 | 41.5 |
| 5720 ^a | 0.000 | 3 | 23.4 |
| 3 mg/kg/day | | | |
| 5702 | 0.000 | 4 | 10.6 |
| 5706 | 0.170 | 3 | 21.8 |
| 5717 | 0.099 | 3 | 22.8 |
| 5721 ^b | 0.000 | NP | 13.0 |
| 10 mg/kg/day | | | |
| 5707 | 0.075 | 3 | 15.5 |
| 5708 | 0.050 | 3 | 17.1 |
| 5710 | 0.024 | 3 | 17.9 |
| 5716 ^a | 0.000 | 4 | 10.9 |
| 5712 ^a | 0.073 | 3 | 18.8 |
| 5719 | 0.025 | 3 | 35.2 |
| 30/20 mg/kg/day | | | |
| 5703 ^c | 0.075 | 3 | 20.2 |
| 5704 | 0.100 | 3 | 10.8 |
| 5711 ^d | 0.126 | 4 | 21.7 |
| 5713 | 0.075 | 4 | 15.8 |
| 5722 ^e | 0.149 | 3 | 14.3 |
| 5724 ^f | ND | 3 | 16.4 |

Note. Liver, only dark nuclear stained hepatocytes scored as S-phase at 200 \times . Pancreas, scored subjectively with 4 = islets and exocrine heavily stained (> 50% labeled) and 3 = exocrine stained > islets (100 \times and 400 \times). Testes, section farthest from slide label, Leydig cells at 400 \times . NP, tissue not present on slide. ND, not determined due to too much nonspecific staining.

^aRecovery animal.

^bSacrificed in moribund condition during Week 20.

^cDosing suspended Week 12.

^dDosing suspended Week 7.

^eDosing suspended Week 10.

^fSacrificed in moribund condition Day 29.

of a 28-day dose range-finding po capsule study in the cynomolgus monkey. In the dose range-finding study, monkeys were administered the test compound at concentrations of 2 and 20 mg/kg for 28 days. The results of this study revealed no changes in any toxicological or biochemical parameter including organ and body weight measurements. As this was only a 28-day study and the serum half-life of elimination of APFO in the monkey was expected to be greater than 1 month, dose levels of 3, 10, and 30 mg/kg were selected for the 6-month study.

Toxicity occurred early in the study at 30 mg/kg/day, requiring suspension of dosing between Days 12 and 22. After reestablishing dosing on Day 22 at 20 mg/kg, three monkeys again displayed signs similar to those that appeared after dos-

ing at 30 mg/kg. As a result, the quantitative interpretation of responses at the high dose in this study becomes complicated. Two monkeys did tolerate 30/20 mg/kg for the 6-month dosing period. In contrast, 10 mg/kg was not associated with untoward effects in any of the 6 monkeys treated. Therefore, a sharp delineation between effect and no effect for these symptoms occurs between 10 and 20/30 mg/kg/day.

The moribund condition of one 30/20 mg/kg/day dose-group monkey that led to an unscheduled sacrifice reflects the relatively high toxicity observed in the first few weeks of dosing at 30 mg/kg in all high dose monkeys. Symptoms seen in this monkey (aphagia, lethargy, dehydration, and severe loss of body weight) were consistent with those seen in other high-dose monkeys that were experiencing distress after dosing at 30 mg/kg. Therefore, it is likely that the moribund condition of this monkey may have been related to APFO treatment; however, the specific cause remains unclear. Although the esophageal and gastric lesions, the degeneration and necrosis of heart tissue, and the dehydration experienced by this monkey are complicating factors, the contribution of APFO to this monkey's moribund condition cannot be dismissed. Findings in the liver and the concomitant elevations of serum alanine aminotransferase, aspartate aminotransferase, and sorbitol dehydrogenase were likely due to APFO exposure.

One could speculate that the extreme elevation in creatinine phosphokinase and the extreme decrease in serum cholesterol along with the necrosis of heart muscle raise the possibility that this monkey may have suffered from rhabdomyolysis and lactic acidosis as a result of reductions in ubiquinone. These findings are consistent with what has been reported for certain HMG CoA reductase inhibitors (statins), especially when given in combination with fibrates (De Pinieux *et al.*, 1996; Omar *et al.*, 2001). Furthermore, one could speculate that the mitochondrial proliferation observed in this study may have been a result of downregulation of HMG CoA reductase by PFOA (Haughom and Spydevold, 1992) leading to a concomitant reduction in ubiquinone leading to decreased oxidative phosphorylation and increased mitochondrial biosynthesis (Berthiaume and Wallace, 2002; Fosslie, 2001). Statins have been shown to decrease ubiquinone and increase the lactate/pyruvate ratio (De Pinieux *et al.*, 1996; Flint *et al.*, 1997).

The *in extremis* condition of the low-dose monkey that was sacrificed on Day 137 is puzzling. This monkey had signs of mild dehydration before initiation of dosing. Normally, this would not be considered a potential confounder; however, one week prior to the unscheduled sacrifice, this monkey exhibited some signs that were seen in the high-dose monkey that was sacrificed early (aphagia, lethargy, dehydration, and severe loss of body weight). In addition, this low-dose monkey had signs of ataxia, restricted hind-limb movement, and an apparent absence of reaction to painful stimuli. No evidence of dosing or cage injury was found. The blood supply to the hind limbs appeared to be severely compromised, although pathological examination did not reveal evidence of spinal cord injury or

impaired circulation. This may suggest a neurogenic response without shock such as alpha agonism. A complete review of the in-life monkey history including review of the clinical and microscopic pathology by a team of pathologists also failed to explain the cause of this monkey's extreme poor health. These findings were nonspecific and, with the exception of low albumin concentration, decreased or no food consumption, and body weight loss, were not consistent with the findings observed for monkeys in the high-dose group that were withdrawn from treatment. It is notable that the liver-to-body weight percentage for this monkey was 2.4, representing a significant increase in liver weight comparable to high-dose monkey values.

Although statistically significant only at the highest dose, there was an indication of increased relative liver weight at all treatment levels. This finding appears to be the most sensitive dose-related effect of APFO observed in this study (Table 6). The increase in liver weights seen following the administration of APFO to cynomolgus monkeys was, at least in part, due to hepatocellular hypertrophy (as demonstrated by decreased hepatic DNA content) which in turn may be explained by mitochondrial proliferation (as demonstrated by increased succinate dehydrogenase activity).

This finding of increased liver weight differs from the findings of a 90-day po gavage study in male and female rhesus monkeys (Griffith and Long, 1980). The rhesus study was conducted with APFO in male and female rhesus monkeys at doses of either 0, 3, 10, 30, or 100 mg/kg/day in 0.5% Methocel. Mortality occurred at doses of 30 and 100 mg/kg. Although an apparent NOAEL of 3 mg/kg was obtained, clinical signs of emesis complicated dosage delivery. A clear determination of a target-organ effect was not obtained in the rhesus study.

A number of special endpoints were selected for inclusion in the study to assist in the determination of potential mechanisms of action. These endpoints were selected primarily because of a suggested link between endpoints observed in long-term rodent bioassays with APFO in which hepatocellular, pancreatic acinar cell, and testicular Leydig cell tumors occurred (Biegel *et al.*, 2001; Pastoor *et al.*, 1987; Riker Pharmaceuticals, 1987).

Changes associated with the potential production of liver tumors in the rat were absent in the cynomolgus monkey. The approximately 2-fold increase in hepatic palmitoyl CoA oxidase activity (a marker for peroxisome proliferation) at the 30/20 mg/kg/day dose (Table 3), is consistent with previous reports for species that are not particularly responsive to peroxisome proliferating compounds (Ashby *et al.*, 1994; Bentley *et al.*, 1993; Cattley *et al.*, 1998). Replicative DNA synthesis, as an indication of cell proliferation, in selected organs (liver, pancreas, and testes) was found to be unaltered in monkeys treated with APFO (Table 7).

Similarly, changes that have been suggested to be associated with the production of pancreatic acinar cell tumors from exposure to peroxisome proliferators in rats include increased

serum cholecystokinin concentrations and indications of cholestasis, including alkaline phosphatase, bilirubin, and bile acids (Obourn *et al.*, 1997). These changes did not occur in the cynomolgus monkeys in this study.

In the rat, APFO has been shown to cause sustained increases in estradiol resulting from aromatase induction that have been related to the occurrence of Leydig cell tumors. Among APFO-treated monkeys in this study, no significant changes in levels of the circulating sex hormones, estrone, estradiol, estriol, or testosterone were observed that could be related to treatment.

The data were analyzed both by intergroup comparisons and monkey-by-monkey using each monkey's pretreatment values as the baseline. As expected, a wide range of values was seen both within a particular monkey and among the group of monkeys, which made evaluation of this information very difficult. When looking at the data for trends, with estradiol as an example, values during the treatment period tended to be lower than those seen in the pretreatment and recovery periods regardless of group assignment. Since some of the lower values fell in the 30/20 mg/kg group, it is tempting to consider this as treatment-related, but an objective viewing of this information finds too little cause/effect to attribute the apparent changes to treatment. The failure to see any histopathologic correlation, while not directly bearing on the issue, tends to support the lack of an APFO-related finding.

The results of this study corroborate the relative lack of response of the cynomolgus monkey to the effects of peroxisome proliferating compounds as observed in rats on liver, pancreas, and testes (Cattley *et al.*, 1998; Kurata *et al.*, 1998; Pugh *et al.*, 2000). Assuming that the cynomolgus monkey response to the effects of APFO is more representative of the human than of the rat, the effects observed in rats may have questionable relevance to humans.

There were no clear changes in thyroid hormone homeostasis. All thyroid hormone values were within normal range, and there did not appear to be any relevant histological changes or changes in T₄ or TSH. However, the three high-dose monkeys that were removed from dosing on Days 44, 66, and 81 had T₃ values that trended down compared to pretreatment measurements, and T₃ appeared to trend back toward prestudy values after cessation of dosing. Therefore, the significance of trends in T₃ in these three high-dose monkeys is unclear. Our conclusion is that these apparent changes are best explained by normal variation or stress and are not a direct effect of APFO on thyroid hormone homeostasis.

Although there were only two 10 mg/kg recovery monkeys, there were no test compound-related effects on terminal body weights or on absolute or relative organ weights, indicating that the liver weight increase seen at termination of the dosing period appeared to be reversible. All other macroscopic, microscopic, and clinical observations were normal.

Serum and liver PFOA concentrations did not appear to increase in linear proportion to dose, possibly due to reaching

saturation and steady state in the first several weeks of the dosing period. The reason for the rather high degree of variability in measured serum PFOA concentrations is not completely understood. This variability may be due, in part, to the capsule method of dosing. Also, since PFOA is believed to be eliminated in the bile (Johnson *et al.*, 1984) and reabsorbed, the timing of dosing, blood sample collection, and gall bladder emptying may have played a role. Another factor that must be taken into consideration is the precision of the method, which was $\pm 30\%$ (interassay, intra-assay, and system). When taken as repeat measures from Week 6 on, the serum concentrations at the two lower dose levels were not statistically different. At the high dose, the concentrations were higher than those at the two lower doses. Similarly, liver PFOA concentrations for monkeys sacrificed at the end of dosing were similar in the 3 and 10 mg/kg dose groups (means of 15.9 and 14.4 $\mu\text{g/g}$ liver, respectively). For the two monkeys in the high-dose group that remained on treatment until sacrifice, the liver concentrations were 16.6 and 86.9 $\mu\text{g/g}$. The lack of linear proportionality to dose of serum concentrations observed in this study was also observed in an earlier 90-day rhesus monkey study (Griffith and Long, 1980). In that study, the male serum concentrations ($n = 1$ per dose group) were reported to be 49, 46, and 145 ppm at doses of 3, 10, and 30 mg/kg/day, respectively. In the same rhesus study, liver concentrations were reported to be 3, 9, and 60 ppm at dose levels of 3, 10, and 30 mg/kg/day, respectively. The clearance of PFOA observed in this study suggests that clearance is more rapid than that suggested for retired workers (Ubel *et al.*, 1980). Additional work is in progress to better understand the toxicokinetics of PFOA in human and nonhuman primates.

The doses that were administered in the study were considerably higher than those that are expected from occupational exposure. The American Conference of Governmental Industrial Hygienists (ACGIH) has established a Threshold Limit Value[®] (TLV[®]) of 0.01 mg/m³ as a time-weighted average exposure for workers (ACGIH, 1999). A 3 mg/kg daily dose from inhalation exposure would require an air concentration of 21 mg/m³, a value considerably above the TLV[®].

The authors acknowledge that the small numbers of monkeys used in the study, their heterogeneity, attrition of monkeys due to toxicity and nontreatment-related causes, the need to reduce the high dose early in the study, and the absence of a clear no-effect level may limit the ability to draw conclusions on some points. However, the study was successful in providing insight into the possible mechanism of liver response in the monkey and defining the external and internal dose parameters associated with early toxic response. The study also clearly demonstrates the dramatic demarcation in dose response between a relatively mild response (liver weight increase at the 3 and 10 mg/kg/day dosage) and serious toxicity (dramatic weight loss and one death at the 30/20 mg/kg/day dosage).

In summary, the dose-response characteristics of APFO in the cynomolgus monkey appear to be quite steep. A dose of 10

mg/kg was well-tolerated over a 6-month dosing period with only a minimal effect (liver weight increase); however, 1 week of dosing at 30 mg/kg produced toxicity, and lowering this dose to 20 mg/kg was only tolerated by two of the 6 high-dose monkeys for the 6-month dosing period. A no observable effect level (NOEL) was not determined in this study. The low dose of 3 mg/kg/day is considered a lowest observable effect level (LOEL), based on the suggestion that liver weight may have been increased at 3 mg/kg/day and the uncertainty as to the role of APFO in the moribund condition of a 3 mg/kg dose-group monkey. Effects that have been associated with the development of pancreatic and testicular toxicity in rats were not observed in this study.

ACKNOWLEDGMENTS

We acknowledge the contributions of Lisa Clemen, Mark Ellefson, and Harold Johnson of the 3M Environmental Laboratory; Dale Aldridge, Johnnie Eighmy, Robert Hall, and Patricia McKee-Pesik of Covance Laboratories; John O'Connor of DuPont Haskell Laboratory; Sandy Eldridge of Pathology Associates International; and Barbara Elcombe and Gus Reid of the University of Dundee. This study was sponsored by 3M and member companies of the Association of Plastic Manufacturers of Europe (APME).

REFERENCES

- ACGIH (1999). Ammonium perfluorooctanoate. In *Documentation of the Threshold Limit Values and Biological Exposure Indices*. Compact disk. American Conference of Governmental Industrial Hygienists, Inc., Cincinnati, OH.
- Ashby, J., Brady, A. M., Elcombe, C. R., Elliott, B. M., Ishmael, J., Odum, J., Tugwood, J. D., Kettle, S., and Purchase, I. F. H. (1994). Mechanistically-based human hazard assessment of peroxisome proliferator-induced hepatocarcinogenesis. *Hum. Exp. Toxicol.* **13**(Suppl. 2), S1-S117.
- Belisle, J. (1981). Organic fluorine in human serum: Natural versus industrial sources. *Science* **212**, 1509-10.
- Bentley, P. I., Calder, I., Elcombe, C. R., Grasso, P., Stringer, D., and Wiegand, H.-J. (1993). Hepatic peroxisome proliferation in rodents and its significance for humans. *Food. Chem. Toxicol.* **31**, 857-907.
- Berthiaume, J., and Wallace, K. B. (2002). Perfluorooctanoate, perfluorooctanesulfonate, and N-ethyl perfluorooctanesulfonamido ethanol: peroxisome proliferation and mitochondrial biogenesis. *Toxicol. Lett.* **129**, 23-32.
- Biegel, L. B., Hurtt, M. E., Frame, S. R., O'Connor, J. C., and Cook, J. C. (2001). Mechanisms of extrahepatic tumor induction by peroxisome proliferators in male CD rats. *Toxicol. Sci.* **60**, 44-55.
- Biegel, L. B., Liu, R. C. M., Hurtt, E., and Cook, J. C. (1995). Effects of ammonium perfluorooctanoate on Leydig cell function: *In vitro*, *in vivo*, and *ex vivo* studies. *Toxicol. Appl. Pharmacol.* **134**, 18-25.
- Bourassa, J., Laine, J., Kruse, M. L., Gagnon, M. C., Calvo, E., and Morisset, J. (1999). Ontogeny and species differentiation in the pancreatic expression and localization of the CCK(A) receptors. *Biochem. Biophys. Res. Commun.* **260**, 820-828.
- Bronfman, M., Inestrosa, N. C. and Leighton, F. (1979). Fatty acid oxidation by human liver peroxisomes. *Biochem. Biophys. Res. Commun.* **88**, 1030-1036.
- Cattley, R. C., DeLuca, J., Elcombe, C., Fenner-Crisp, P., Lake, B. G., Marsman, D. S., Pastoor, T. A., Popp, J. A., Robinson, D. E., Schwetz, B., Tugwood, J. and Wahli, W. (1998). Do peroxisome proliferating compounds

- pose a hepatocarcinogenic hazard to humans? *Regul. Toxicol. Pharmacol.* **27**, 47–60.
- Clegg, E. D., Cook, J. C., Chapin, R. E., Foster, P. M. D., and Daston, G. P. (1997). Leydig cell hyperplasia and adenoma formation: Mechanisms and relevance to humans. *Reprod. Toxicol.* **11**, 107–121.
- Cook, J. C., Klinefelter, G. R., Hardisty, J. F., Sharpe, R. M., and Foster, P. M. (1999). Rodent leydig cell tumorigenesis: A review of the physiology, pathology, mechanisms, and relevance to humans. *Crit. Rev. Toxicol.* **29**, 169–261.
- Cook, J. C., Murray, S. M., Frame, S. R., and Hurtt, M. E. (1992). Induction of Leydig cell adenomas by ammonium perfluorooctanoate: A possible endocrine-related mechanism. *Toxicol. Appl. Pharmacol.* **113**, 209–217.
- De Pinieux, G., Chariot, P., Ammi-Said, M., Louarn, F., Lejonec, J. L., Astier, A., Jacotot, B., and Gherardi, R. (1996). Lipid-lowering drugs and mitochondrial function: Effects of HMG-CoA reductase inhibitors on serum ubiquinone and blood lactate/pyruvate ratio. *Br. J. Clin. Pharmacol.* **42**, 333–337.
- DuPont Haskell Laboratory (1982). Unpublished report—Excretion and disposition of ¹⁴C- ammonium perfluorooctanoate in male and female rats, mice, hamsters, and rabbits. U.S. EPA Public Docket AR-226.
- Eldridge, S. R., Butterworth, B. E., and Goldsworthy, T. L. (1993). Proliferating cell nuclear antigen: A marker for hepatocellular proliferation in rodents. *Environ. Health Perspect.* **101**(Suppl. 5), 211–218.
- Flint, O. P., Masters, B. A., Gregg, R. E., and Durham, S. K. (1997). HMG coA reductase inhibitor-induced myotoxicity: Pravastatin and lovastatin inhibit the geranylgeranylation of low-molecular-weight proteins in neonatal rat muscle cell culture. *Toxicol. Appl. Pharmacol.* **145**, 99–100.
- Fosslien, E. (2001). Mitochondrial medicine—molecular pathology of defective oxidative phosphorylation. *Ann. Clin. Lab. Sci.* **31**, 25–67.
- Griffith, F. D., and Long, J. E. (1980). Animal toxicity studies with ammonium perfluorooctanoate. *Am. Ind. Hyg. Assoc. J.* **41**, 576–583.
- Guy, W. S., Taves, D. R., and Brey, W. S. (1976). Organic fluorocompounds in human plasma: Prevalence and characterization. In *Biochemistry Involving Carbon-Fluorine Bond* (R. Filler, Ed.), ACS Symposium Series No. 28, Vol. 7, pp. 117–134. American Chemistry Society, Washington, DC.
- Haugthom, B., and Spydevold, O. (1992). The mechanism underlying the hypolipidemic effect of perfluorooctanoic acid (PFOA), perfluorooctane sulphonic acid (PFOSA) and clofibrate. *Biochim. Biophys. Acta* **1128**, 65–72.
- Hansen, K. J., Clemen, L. A., Ellefson, M. E., and Johnson, H. O. (2001). Compound-specific, quantitative characterization of organic fluorochemicals in biological matrices. *Environ. Sci. Technol.* **35**, 766–770.
- Holicky, E. L., Hadac, E. M., Ding, X. Q., and Miller, L. J. (2001). Molecular characterization and organ distribution of type A and B cholecystokinin receptors in cynomolgus monkey. *Am. J. Physiol. Gastrointest. Liver Physiol.* **281**, G507–G514.
- Intrasukri, U., Rangwala, S. M., O'Brien, M., Noonan, D. J., and Feller, D. R. (1998). Mechanisms of peroxisome proliferation by perfluorooctanoic acid and endogenous fatty acids. *Gen. Pharmacol.* **31**, 187–197.
- Johnson, J. D., Gibson, S. J., and Ober, R. E. (1984). Cholestyramine-enhanced fecal elimination of carbon-14 in rats after administration of ammonium [¹⁴C]perfluorooctanoate or potassium [¹⁴C]perfluorooctanesulfonate. *Fundam. Appl. Toxicol.* **4**, 972–976.
- Johnson, J. D., and Ober, R. E. (1980). Extent and route of excretion and tissue distribution of total carbon-14 in male and female rats after a single IV dose of FC-143-¹⁴C. Riker Laboratories, Inc., St. Paul, MN. U.S. EPA Public Docket AR-226.
- Kawashima, Y., Kobayashi, H., Miura, H., and Kozuka, H. (1995). Characterization of hepatic responses of rat to administration of perfluorooctanoic and perfluorodecanoic acids at low levels. *Toxicology* **99**, 169–178.
- Kennedy, G. L., Jr. (1987). Increase in mouse liver weight following feeding of ammonium perfluorooctanoate and related fluorochemicals. *Toxicol. Lett.* **39**, 295–300.
- Kurata, Y., Kidachi, F., Yokohama, M., Toyota, N., Tsuchitani, M., and Katoh, M. (1998). Subchronic toxicity of Di(2-ethylhexyl)phthalate in common marmosets: Lack of hepatic peroxisome proliferation, testicular atrophy, or pancreatic acinar cell hyperplasia. *Toxicol. Sci.* **42**, 49–56.
- Kuslikis, B. I., Vanden Heuvel, J. P., and Peterson, R. E. (1992). Lack of evidence for perfluorodecanoyl- or perfluorooctanoyl-coenzyme A formation in male and female rats. *J. Biochem. Toxicol.* **7**, 25–29.
- Liu, R. C. M., Hahn, C. and Hurtt, M. E. (1996a). The direct effect of hepatic peroxisome proliferators on rat Leydig cell function *in vitro*. *Fundam. Appl. Toxicol.* **30**, 102–108.
- Liu, R. C. M., Hurtt, M. E., Cook, J. C., and Biegel, L. B. (1996b). Effect of the peroxisome proliferator, ammonium perfluorooctanoate (C8), on aromatase activity in tissues of male Crl:CD BR (CD) rats. *Fundam. Appl. Toxicol.* **30**, 220–228.
- Maloney, E. K., and Waxman, D. J. (1999). Trans-activation of PPAR α and PPAR γ by structurally diverse environmental chemicals. *Toxicol. Appl. Pharmacol.* **161**, 209–218.
- Obourn, J. D., Frame, S. R., Bell, R. H., Jr., Longnecker, D. S., Elliott, G. S., and Cook, J. C. (1997). Mechanisms for the pancreatic oncogenic effects of the peroxisome proliferators Wyeth-14,634. *Toxicol. Appl. Pharmacol.* **145**, 425–436.
- Olsen, G. W., Burris, J. M., Burlew, M. M., and Mandel, J. H. (2000). Plasma cholecystokinin and hepatic enzymes, cholesterol and lipoproteins in ammonium perfluorooctanoate production workers. *Drug Chem. Toxicol.* **23**, 603–620.
- Olsen, G. W., Gilliland, F. D., Burlew, M. M., Burris, J. M., Mandel, J. S., and Mandel, J. H. (1998). An epidemiologic investigation of reproductive hormones in men with occupational exposure to perfluorooctanoic acid. *J. Occup. Environ. Med.* **40**, 614–622.
- Omar, M. A., Wilson, J. P., and Cox, T. S. (2001). Rhabdomyolysis and HMG-CoA reductase inhibitors. *Ann. Pharmacother.* **35**, 1096–1107.
- Pastoor, T. P., Lee, K. P., Perri, M. A., and Gillies, P. J. (1987). Biochemical and morphological studies of ammonium perfluorooctanoate-induced hepatomegaly and peroxisome proliferation. *Exp. Mol. Pathol.* **47**, 98–109.
- Permadi, H., Lundgren, B., Anderson, K., and DePierre, J. W. (1992). Effects of perfluoro fatty acids on xenobiotic-metabolizing enzymes which detoxify reactive forms of oxygen and lipid peroxidation in mouse liver. *Biochem. Pharmacol.* **44**, 1183–1191.
- Permadi, H., Lundgren, B., Andersson, K., Sundberg, C., and DePierre, J. W. (1993). Effects of perfluoro fatty acids on peroxisome proliferation and mitochondrial size in mouse liver: Dose and time factors and effect of chain length. *Xenobiotica* **23**, 761–770.
- Pugh, G., Jr., Isenberg, J. S., Kamendulis, L. M., Ackley, D. C., Clare, L. J., Brown, R., Lington, A. W., Smith, J. H., and Klaunig, J. E. (2000). Effects of di-isononyl phthalate, di-2-ethylhexyl phthalate, and clofibrate in cynomolgus monkeys. *Toxicol. Sci.* **56**, 181–188.
- Reddy, J. K., and Rao, M. S. (1977). Malignant tumors in rats fed nafenopin, a hepatic peroxisome proliferator. *J. Natl. Cancer Inst.* **59**, 1645–1650.
- Riker Pharmaceuticals (1987). Two year oral (diet) toxicity/carcinogenicity study of fluorochemical FC-143 in rats. Riker Laboratory, Experiment number 0281CR0012. U.S. EPA Public Docket AR-226.
- Ubel, F. A., Sorenson, S. D., and Roach, D. E. (1980). Health status of plant workers exposed to fluorochemicals—a preliminary report. *Am. Ind. Hyg. Assoc. J.* **41**, 584–589.
- Vanden Heuvel, J., Kuslikis, B., Van Refelghem, M., and Peterson, R. (1991). Tissue distribution, metabolism, and elimination of perfluorooctanoic acid in male and female rats. *J. Biochem. Toxicol.* **6**, 83–92.