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## Toxicological and Pathological Studies with 3,3-Dimethyl-1-Phenyltriazene: with Special Reference to Hepatic Damage.* (23517)

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During routine screening of compounds for anti-tumor effects, 3, 3-dimethyl-1-phenyltriazene was found to inhibit Sarcoma 180 in mice (1). The agent was also shown to be teratogenic in chick embryos(2) and to prolong the survival of mice bearing Leukemias 82 and 5471(3). Since this triazene was representative of a new series of compounds not previously known to be active against experimental tumors, it became of interest for possible clinical trial. For this reason pharmacological studies were undertaken in our laboratories.

*Procedure.* The kinds of animals used and the procedures employed have recently been described(4,5). The triazene, a liquid, was dissolved in peanut oil and 0.5 ml of the solution per 100 g of body weight was given intraperitoneally to mice and rats. For oral administration to rodents the agent was suspended in 0.9% NaCl containing 0.5% carboxymethylcellulose (CMC). For intravenous injections the compound was dissolved in propylene glycol while for intramuscular injection it was diluted with peanut oil. Dogs received the agent orally in gelatin capsules. $LD_{50}$ values were calculated according to Litchfield and Wilcoxon(6) after administration of a sufficient number of doses, decreasing in series by a factor of ½, to obtain both 100 and 0% mortality during 14 days of observation. At least 10 mice, 6 rats, 2 cats, and 2 dogs were employed per dose.

*Results. Toxicity in rodents.* Table I is a summary of toxicity data. The compound was somewhat more toxic to mice and rats when given intraperitoneally than when given orally. Within 2 hours after 500 mg/kg intraperitoneally or 1000 mg/kg orally the righting reflexes of mice became impaired; a progressing depression ensued and death occurred between 3 and 20 hours. Following single doses of 250 mg/kg intraperitoneally or 500 mg/kg orally mice became depressed, dyspneic, and ataxic within 3 to 4 hours. A few died within the first day; most of the remainder between the second and eleventh days. The majority of those alive at 24 hours moved about in circles and their heads bobbed continuously from side to side. This syndrome, presumably neurologic in origin, was further characterized by the inability of the mice, when placed in water, to swim in a well-coordinated manner. The disturbance seemed similar to that seen in congenitally abnormal

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TABLE I. Toxicity of 3,3-Dimethyl-1-phenyltriazene.

| Species | Route of admin. | No. of successive daily doses | $LD_{50}$,* mg/kg/day | 19/20 confidence limits,* mg/kg/day | S* |
|---|---|---|---|---|---|
| Mice | Intraper. | 1 | 200 | 160–250 | 1.30 |
| | " | 5 | 72 | 30–183 | 1.52 |
| | Oral | 1 | 290 | 200–420 | 1.82 |
| Rats† | Intraper. | 1 | 180 | 136–238 | 1.42 |
| | " | 5 | 71 | 64– 78 | 1.10 |
| | Oral | 1 | 180 | 333–608 | 1.35 |
| | " | 5 | 130 | 86–196 | 1.55 |
| | | | Dose, mg/kg/day | No. dead/No. tested | Day of death |
| Cats | Intrav. | 1 | 100 | 2/2 | Immediate |
| | | | 50 | 0/2 | |
| | | | 25 | 0/2 | |
| | Intramuse. | 1 | 200 | 2/2 | 1, 1 |
| | | | 100 | 1/2 | 3 |
| | | | 50 | 0/2 | |
| Dogs | " | 10‡ | 50 | 2/2 | 10, 18 |
| | | | 25 | 1/2 | 26 |
| | | | 12.5 | 0/2 | |
| | | | 6.25 | 0/2 | |
| | Oral | 10‡ | 50 | 1/2 | 35 |
| | | | 25 | 0/2 | |
| | | | 12.5 | 0/2 | |

* Calculated according to Litchfield and Wilcoxon(6). Animals were observed for at least 2 wk after the end of treatment.

† Carworth Farms Wistar (CFW).

‡ Maximum No. of doses given daily except weekends.

"waltzing" or "shaker" mice(7); the same syndrome has been induced by the administration of certain mono-2-chloroethylamines (8). Except for the absence of the neurological disturbance, the course of intoxication in rats given single lethal doses was like that seen in mice. When given on each of 5 successive days, the triazene caused progressive weight loss, ataxia, dyspnea, and depression in both mice and rats. At doses of 100 to 125 mg/kg/day intraperitoneally and 100-200 mg/kg/day orally deaths occurred between the sixth and eleventh days after the initiation of treatment. Pathological studies were not performed.

*Pathological changes in rats.* Lesions were studied in a group of 26 Carworth Farms Nelson (CFN) rats which received 100 mg/kg day of the triazene in a maximum of 5 successive daily injections. Six animals were sacrificed at 2 days (24 hours after the second injection); at this time the rats appeared unkempt. On the fourth day (24 hours after the fourth injection) 6 additional animals were sacrificed; weight loss and "bloody" exudates about the nares were evident. Six rats were sacrificed on the seventh day (72 hours after the fifth injection); further weight loss had occurred and the rats appeared unkempt and had "bloody" nares. Four of the remaining 8 animals died on days 8, 16, 21, and 21, respectively. Control rats which had received injections of peanut oil were also sacrificed: at 2, 4, and 7 days. Significant hematological changes occurred during treatment (Table II). By the end of the second day reticulopenia and leukopenia were already evident; by the fourth day these changes were even greater and they were accompanied by a fall in the hematocrit. At the seventh day the reticulocyte count had returned to normal values but the leukopenia and anemia were still evident. On *gross* examination the following were noted in all treated animals: an "oily" abdominal ascites (present also in controls), semi-fluid or fluid bone marrow from the femoral shaft, and small thymi. At 4 and 7 days pallor of the inner cortex of the kidney was also prominent. Microscopic examination revealed lesions in a number of tis-

TABLE II. Weight and Blood Cells in Rats Treated with 3,3-Dimethyl-1-phenyltriazene. (Mean values ± stand. dev.)

| Day of sacrifice | Wt change (%) | Reticulocytes (%) | Hematocrit (ml/100 ml) | Platelets ($10^3$ cells/mm$^3$) | WBC* ($10^3$ cells/mm$^3$) |
|---|---|---|---|---|---|
| Control† | + 5 ± 5 | 3.1 ± 1.1 | 46.3 ± .9 | 437 ± 210 | 5.61 ± 1.7 |
| 2 days | − 3 ± 4 | .6 ± .6 | 46.0 ± 3.3 | 506 ± 127 | 1.04 ± .5 |
| 4 | −17 ± 3 | .01 ± .04 | 42.0 ± 4.3 | 602 ± 172 | .11 ± .1 |
| 7 | −29 ± 3 | 4.0 ± 3.6 | 29.0 ± 5.4 | 503 ± 160 | .05 ± .1 |

* White blood cells. † Avg of 12 animals, 4 at each of days 2, 4, and 7.

sues as early as 2 days after initiation of treatment. These progressed in severity and in all 6 animals sacrificed at 7 days they were as follows: the lymphocytic elements of spleen, thymus, and nodes were markedly depleted; the red pulp of the spleen was congested and the extra-medullary hematopoietic cells were reduced in number; the sternal bone marrow showed a 90 per cent loss of nucleated elements; spermatogenic arrest was observed in some of the testicular tubules and this was associated with multinucleated giant cells; in the kidneys there was necrosis in the distal portion of the proximal convoluted tubules as well as a mild membranous glomerulonephritis; a peripancreatic inflammatory reaction was present. The livers appeared normal at 2 and 4 days but at 7 days showed a loss of cytoplasmic basophilia and a slight enlargement of hepatic cells in central zones. Hepatic necrosis was not evident. Finally, at 2 and at 4 days the mucosa of the small bowel had some atypia manifested by nuclear swelling and loss of polarity of the inner mucosal glands; by 7 days the small intestine showed no abnormalities.

*Toxicity in cats.* Table I presents the toxicity data for triazene in cats. The pair given 100 mg/kg intravenously showed immediate respiratory arrest. Cats given 200 mg/kg intramuscularly died unobserved overnight; one was dyspneic for several hours after injection while the other became ataxic after 4 hours and developed paralysis of the hind legs. Of the pair given 100 mg/kg intramuscularly one developed ataxia of the hind legs by 10 hours. This was no longer present at 24 hours and the cat survived. The other animal died at 3 days; it was markedly ataxic, dyspneic, and cyanotic on the day after injection and exhibited episodes of tonic and clonic convulsions. No remarkable changes were noted in animals receiving 50 mg/kg intravenously or intramuscularly. Pathological studies were not performed.

*Pathological changes in dogs.* The toxicity data in dogs are given in Table I. In the 8 animals given 25 or 50 mg/kg/day anorexia, weight loss, and emesis became evident during the first week of treatment. Six of this group became jaundiced during treatment or within 1 week thereafter. Dilute urine (specific gravity $\leq$ 1.015) was also observed in 6 of the 8 animals. Body temperatures remained relatively normal throughout the course of fatal intoxications. To determine the lesions associated with intoxication 4 additional dogs were given the doses described in Table III and sacrificed at different times during or after treatment. As seen in Table III, each animal lost weight during treatment. Dogs 2 and 4 showed a decrease in the hematocrits without concomitant decrease in reticulocyte counts. Lymphocytopenia became evident in 3 dogs but there were no consistent changes in the counts of neutrophilic granulocytes. Thrombocytopenia developed in dogs 3 and 4 though blood clotting time was not significantly affected. In dogs 3 and 4 there developed prolonged prothrombin times and severe deficiencies in the fibrinogen content of plasma. This evidence of hepatic insufficiency was associated with hyperbilirubinemia and increased sulfobromophthalein (BSP) retention. In addition the activity of plasma alkaline phosphatase rose in each animal and especially in dog 4. The total serum protein decreased in all animals. In other biochemical analyses, which are not recorded in Table III, plasma non-protein nitrogen and blood glucose remained unchanged while decreases were observed in plasma chloride to as low as 70 meq/l at day 15 in dog 3.

At autopsy the tissues of the 3 dogs given

TABLE III. Hematological and Biochemical Data from Dogs Treated with 3,3-Dimethyl-1-phenyltriazene.

| Dog | Day | Wt (kg) | Herit (ml/100 ml) | Retic (%) | Leyte ($10^3$ cells/$mm^3$) | Pltlt | Prot (g %) | Prothb time (sec.) | Blrbn | BSP | Alk ptase | Fbgn |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | (mg %) | | | |
| 1 | 50 mg/kg × 5, IM | | | | | | | | | | | |
| | 0 | 14.8 | 52.5 | tr | 2.6 | 208 | 5.9 | 13.9 | .3 | .2 | .5 | |
| | 4 | 12.9 | 53 | .3 | .1 | | 5.3 | 13.8 | 1.0 | 1.4 | 1.5 | |
| | 7 *S* | 12.7 | 52 | .3 | .1 | 120 | 5.1 | 14.7 | 8.3 | 3.5 | 3.3 | |
| 2 | 50 mg/kg × 7, PO | | | | | | | | | | | |
| | 0 | 10.3 | 53 | 1.0 | 3.7 | 192 | 5.5 | 13.4 | .2 | .3 | 2.2 | |
| | 7 | 9.1 | 41.5 | .6 | .3 | 174 | 5.1 | 10.3 | .5 | 1.1 | 4.8 | |
| | 9 *S* | 8.7 | 39 | 1.4 | .1 | 180 | 4.4 | 10.9 | .8 | 1.6 | 9.8 | |
| 3 | 25 mg/kg × 10, IM | | | | | | | | | | | |
| | 0 | 14.8 | 54 | .1 | 3.0 | 158 | 5.8 | 13.9 | .2 | .2 | 1.0 | 233 |
| | 8 | 13.1 | 53 | .2 | 1.5 | 280 | 5.1 | 20.5 | .7 | 2.0 | 5.0 | 218 |
| | 15 *S** | 12.1 | 51 | 1.2 | .7 | 46 | 2.9 | 45 | 11.8 | 4.0 | 6.7 | † |
| 4 | 25 mg/kg × 10, IM | | | | | | | | | | | |
| | 0 | 11.1 | 52 | .2 | .8 | 176 | 5.0 | 12.3 | .2 | .2 | .8 | 234 |
| | 8 | 9.9 | 45 | .8 | .5 | 278 | 5.0 | 12.6 | .7 | .8 | 2.3 | 363 |
| | 15 | 8.7 | 46 | 2.6 | .6 | 76 | 4.4 | 17.9 | 4.5 | 3.0 | 6.2 | 226 |
| | 18 | 8.5 | | | | | | 32 | | | | |
| | 22 | 8.2 | 43 | 1.5 | .9 | 10 | 4.2 | 22.9 | 3.9 | 2.7 | 19.9 | 110 |
| | 30 | 8.5 | 49 | .5 | .6 | 46 | 4.4 | 19.6 | 1.7 | 3.2 | 20.3 | † |
| | 32 *S* | 8.7 | 45 | .3 | .9 | 42 | | 19.8 | 1.2 | 2.6 | 19.8 | 180 |

Abbreviations: Alk ptase, plasma alkaline phosphatase; Blrbn, total plasma bilirubin; BSP, sulfobromophthalein retention in blood; Fbgn, plasma fibrinogen; Herit, hematocrit; Leyte, lymphocytes; Pltlt, platelets; Prot, total plasma protein; Prothb time, one-stage prothrombin; Retic, reticulocytes; IM, intramuse.; PO, oral.

* Dog 3 was moribund at sacrifice.

† The oxalated plasma failed to clot upon addition of $CaCl_2$ and thrombin.

the triazene intramuscularly were jaundiced and the lobular markings of their livers were accentuated. The liver of dog 3 appeared to be fatty. This animal as well as dog 4 had clear yellow ascitic fluid in the abdomen. Except for minute hemorrhages in the pylorus of the stomach and bloody mucus in the ileum of dog 2, which had received the oral dose, there were no other noteworthy gross findings. On *microscopic* examination the most prominent changes were found in the livers of all 4 dogs. These are described in detail because the lesions varied considerably in the different individuals and because the hepatotoxic effects appeared to be novel. Dog 1 showed damage in the central zone affecting approximately one-third of the lobule. In the involved portions the majority of the hepatic cells were small and there was disorganization of their cord-like arrangement. Loss of cell boundaries was frequent and cytoplasmic vacuolization was evident. A few deeply eosinophilic hepatic cells were present intermixed with polymorphonuclear leukocytes. Canaliculi showed bile-plugging in all portions of the lobule but most prominently in central zones. Some medium-sized bile ducts contained inspissated bile and in these there was some loss of nuclear polarity in the epithelium. The liver of dog 2 had changes that were similar except for involvement of only about one-fourth of the lobule and less prominent plugging of the bile canaliculi. On the other hand more diffuse and extensive effects were observed in the livers of dogs 3 and 4, which were sacrificed at later times, 15 and 34 days, respectively. It is interesting to recall that the plasma prothrombin times became markedly elevated in dogs 3 and 4 after the end of the first week of treatment (Table III). Nearly all liver cells were larger and more varied in shape than normal. The cord-like arrangement was lost resulting in disorganization of the lobular pattern. In the central zones the cytoplasm of most liver cells was finely vacuolated while in peri-portal regions it was deeply eosinophilic. Many cells contained enlarged and irregular nuclei and nu-

cleoli, prominently in the periphery of the lobule. Necrotic cells containing eccentric nuclei and dark pink cytoplasm were scattered throughout the liver; some of these were accumulated in small nests. In dog 3 there was plugging of canaliculi and inspissation of bile with loss of polarity in the epithelium of the medium ducts. No inflammatory reaction was observed. In dog 4 although canalicular stasis was minimal, bile-laden macrophages were present around the central veins intermixed with red cells, polymorphonuclear leukocytes, and lymphocytes.

The other lesions seen are briefly recorded. The bone marrows of all 4 dogs were congested; but only in dog 1 was there found a slight diminution in the number of nucleated elements. In dogs 3 and 4 the sinuses of the lymph nodes contained red cells and acute ulcers were present in the tonsils. Recent ulcers were also found in the buccal mucosa and esophagus of dog 3. In the same animal small hemorrhages were present in the adrenals and colon and the small intestines were congested; interstitial edema of the pancreas was noted with occasional inflammatory cells and some disorganization of the acinar pattern; spermatogenic arrest and numerous giant cells were seen in the testes. In dog 4 necrotic cells were found in the ovarian follicles; a membranous glomerulonephritis was present in its kidneys as well as a solitary focus of acute pyelonephritis.

*Discussion.* The present study has revealed the major pathologic changes induced by 3, 3-dimethyl-1-phenyltriazene in rats and dogs. The peripheral blood, the bone marrow, and the kidney were the primary sites of abnormality in the rat. In dogs the principal pathologic changes were confined to the liver. These included abnormalities in the size and shape of hepatic cells, necrosis of hepatic cells, disorganization of the lobular pattern, and intrahepatic biliary stasis. Biochemical changes indicative of hepatic dysfunction were associated with the liver lesion, namely, increases in the one-stage prothrombin time, hyperbilirubinemia, elevations in the retention of sulfobromophthalein and in the activity of serum alkaline phosphatase, and decreases in the fibrinogen content of plasma.

The contrast in the sites of action in the two species was probably not an absolute one; for hematologic changes such as thrombocytopenia and a slight hypoplasia of bone marrow were noted in a few dogs and a membranous glomerulonephritis was seen in one. Conversely intoxicated rats evidenced minor alterations in the hepatic parenchyma.

The mode of action of the triazene may be related to certain of its chemical properties. Diazoamino compounds are cleaved by acid catalysis to amines and diazonium ions; in the present instance the products would be dimethylamine and benzenediazonium cations. The amine probably has little effect in the amounts formed; but the diazonium moiety could either react directly with cellular constituents or undergo further toxic transformations. For example, hydrolysis would result in phenol while mild reduction would give rise to phenylhydrazine(9). At physiologic pH the cleavage of the triazene would probably be slow, thereby permitting distribution of intact molecules throughout the tissues. If their distribution were non-uniform, varying amounts of toxic moieties would be formed at different sites resulting in selective tissue damage.

In spite of these likely possibilities it has not been possible to explain precisely all of the effects of the present agent in terms of the known properties of the toxic products just mentioned. For example, the anemia in rats and the reticulocytosis and jaundice in dogs seemed suggestive of the hemolytic effects of phenylhydrazine(10). Since the formation of damaged erythrocytes by simple benzene derivatives as well as by phenylhydrazine may be characterized by the presence of Heinz bodies, these abnormalities were sought in the red cells of a pair of dogs given 50 mg/kg/day of the triazene for 5 days (a lethal dose). Smears of daily blood samples, stained by Webster's technic (11), failed to reveal any specific red cell alteration. Since phenol is acutely toxic to the central nervous system and produces convulsions in mice, rats, and cats(12), its formation seemed suggested in the "neurological" syndrome caused by the present agent in mice. Nevertheless, the responses of mice to equimolar, lethal,

single doses of phenol given intraperitoneally were readily distinguished from those caused by the triazene. Moreover, the latter substance was not convulsant in rats or cats.

On the other hand the hepatotoxic and nephrotoxic effects of the triazene may be more readily related to possible transformations *in vivo;* for both phenol(13) and phenylhydrazine(14) have been reported to cause liver and kidney damage in laboratory animals. In this connection it is of interest to note that Kirby (15) has found lesions in liver, spleen, and kidney following the administration of 1,3-diphenyltriazene. This author suggested that the hepatic effects were not due to an *in vivo* rearrangement to *p*-aminodiazobenzene since the latter produces a lesion different from that caused by diphenyltriazene. It is conceivable that the diphenyl derivative may undergo cleavage *in vivo,* as suggested in the case of the agent used herein, with the formation of benzenediazonium cations and aniline.

*Summary.* The toxicity of 3,3-dimethyl-1-phenyltriazene has been described in various laboratory mammals. The agent produced a "neurological" disturbance in mice resembling that seen in the congenitally abnormal "waltzing" or "shaker" strains. In rats lesions were prominent in hematopoietic tissues and in the kidney. In dogs an unusual hepatic lesion was induced which was associated with a variety of disturbances in liver function. The effects of the compound were discussed with respect to possible transformation *in vivo* into phenyldiazonium and various derivatives of the latter substance. Hepatic insufficiency, renal damage, and hematopoietic depression should be anticipated in clinical trials with the agent.

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