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CIGARETTE SMOKE/ETHANOL-INDUCED LIMB DEFECTS IN MOUSE EMBRYOS

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ABSTRACT

We have produced a spectrum of limb defects in developing mouse embryos by exposing the mother to smoke from cigarettes with different nicotine concentrations, ethanol, and a combination of ethanol and cigarette smoke. The critical time of exposure was determined to be during day 10 of gestation (vaginal plug=day 0). This time is prior to the critical events which occur between the apical ectodermal ridge and the developing limb mesenchyme. When pregnant animals were exposed to smoke from two high-nicotine cigarettes (at 10:30 a.m. and 11:30 a.m. on day 10), no limb defects were observed. If ethanol was given (0.015 mg/g 25% i.p. in two doses on the morning of day 10) one percent of offspring showed a limb defect. By contrast, exposure to a combination of cigarette smoke and ethanol resulted in 43% (44/102) of newborns developing both fore - and hindlimb defects. Birth weight was reduced by about 33% in animals carrying the defects. When mesenchyme cells beneath the limb apical ectodermal ridge were examined two days after teratogen exposure, striking changes in cell shape and size were evident. In as much as the incidence of mesenchymal changes in the limb buds seen on day 12 parallels the incidence of malformations seen in newborns, we postulate that anomalous limb development is secondary to the events occurring in the limb mesenchyme. We conclude that critical stages of development occur in the limb buds, and therefore certain teratogens or combinations of teratogenic agents may interfere with their normal development.

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INTRODUCTION

During the past few decades, it has become increasingly evident that human embryos are subject to a variety of environmental influences that might have deleterious effects on their development. Since the thalidomide tragedy, attention has been focused on drugs or chemicals as potential teratogens to which pregnant women might be exposed.[34,39] Two agents to which many women may be exposed are ethanol and smoke from tobacco products. The teratogenic effects of these agents on limb development have received scant attention as yet.

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Nicotine and cigarette smoke

It has been shown that nicotine can cross the placental membrane, causing fetal levels of nicotine to increase rapidly, remain at a steady level, and then slowly decrease as nicotine re-enters the maternal circulation.[52,54,58]

Since 1908 a number of clinical reports concerned with the effects of tobacco or nicotine on the outcome of pregnancy

have appeared.[4,5,10,31,46] Natural abortions were reported to be more prevalent in smokers and in those who worked in tobacco factories than among women employed in other industries.[1,19] Infant mortality rates and the incidence of gynecologic and obstetric complications were higher among smoking mothers.[49,50] It has been shown that smoking during pregnancy is associated with lower birth weight: infants born to women who smoke cigarettes during pregnancy weigh an average of approximately 200 grams less than infants born to non-smoking women.[7,8] As long ago as 1935 Sontage and Wallace, following repeated observations on four subjects, showed that inhalation of cigarette smoke is followed by an increase in the fetal heart rate.[51]

There have been relatively few studies on the teratogenic effects of nicotine or tobacco products in animals. Most studies conducted in this area have involved the chick embryo and thus may not be completely relevant from the point of view of mammalian species. However, these studies do suggest the potential teratogenicity of nicotine.[17] The most common abnormality produced by nicotine in chick embryos was a shortening and twisting of the neck resulting from abnormal development and irregular fusion of the cervical vertebrae.[14,33,57]

Nicotine is also teratogenic in the mammalian embryo. One of the earlier studies in mammalian species was conducted by Hishimura and his group.[23] Another group of investigators has shown that administration of nicotine (95 to 100 µg/kg body weight) to pregnant rats during the initial five days of pregnancy modifies the rate of embryonic cell proliferation, time of implantation, and the time of onset of parturition.[20]

Alcohol

Studies in humans and animals showed that after placental formation, alcohol passed from mother to fetus and the fetal concentration of alcohol approached the maternal concentration. In the fetus, alcohol is distributed in the amniotic fluid, placenta, liver, pancreas, kidney, lung, thymus, brain and heart.[24,25,40]

The possible teratogenic effects of alcohol have been suspected for centuries. It was not until 1968 that Lemoine and coworkers described a pattern of well-defined anomalies in 127 children born to chronic alcoholic mothers.[35] Five years later, Jones and Smith reported a similar pattern of anomalies in the offspring of another group of chronic alcoholic women.[26] This pattern of defects was called the "fetal alcohol syndrome". Since then, many additional cases of fetal alcohol syndrome have been observed.[36,47,52,60]

Smoking and ethanol

Generally, people who drink heavily also tend to smoke heavily. The possibility that the combination of smoking and drinking may interact to produce a greater impact on the developing fetus than either substance alone is suggested by

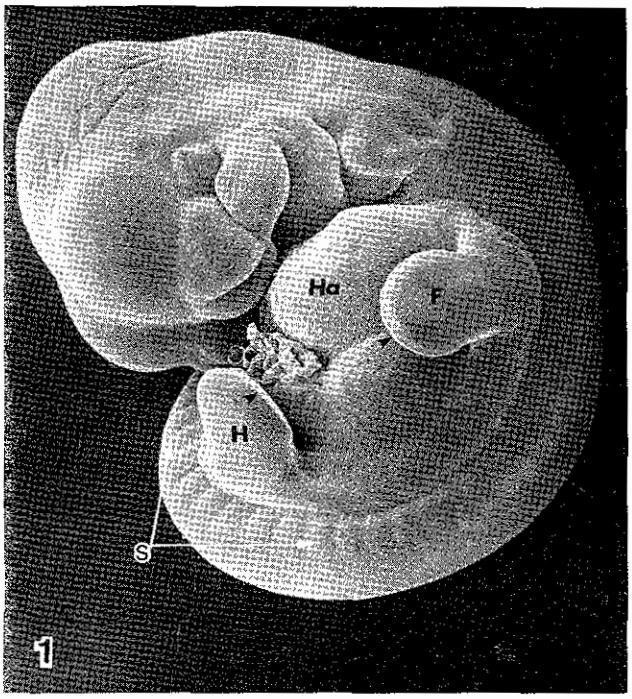


Fig. 1. Lateral view of an embryo on late day 12 of gestation. Arrowheads indicate apical ectodermal ridge. Ha= heart, F = forelimb, H = hindlimb, S = somites.

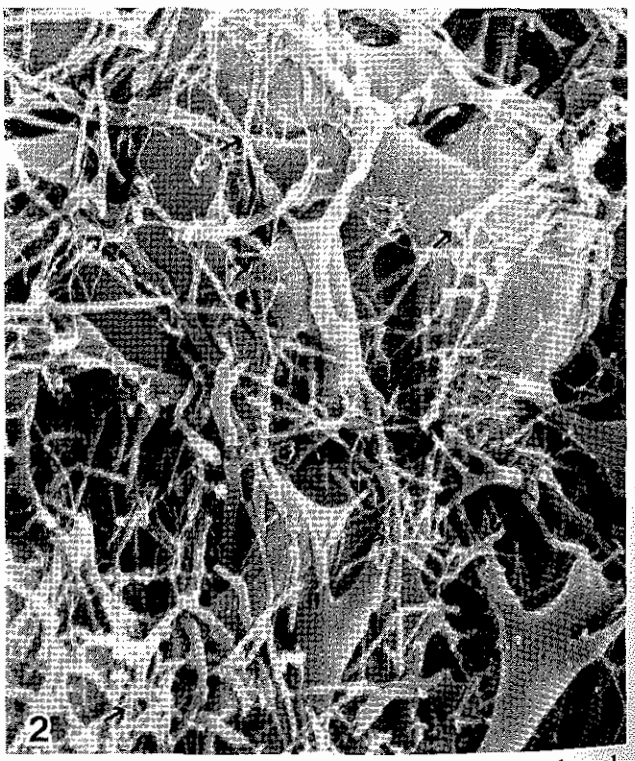


Fig. 2. Dense meshwork of processes (arrows) of mesenchymal cells beneath the apical ectodermal ridge of both fore- and hindlimbs from control embryo, late on day 11 of gestation (44 pairs of somites).

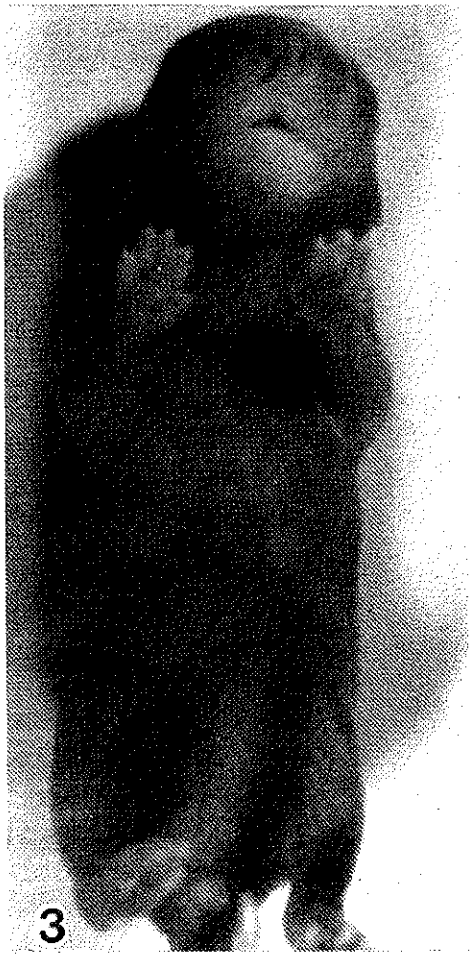


Fig. 3. Anterior view of control newborn mouse.

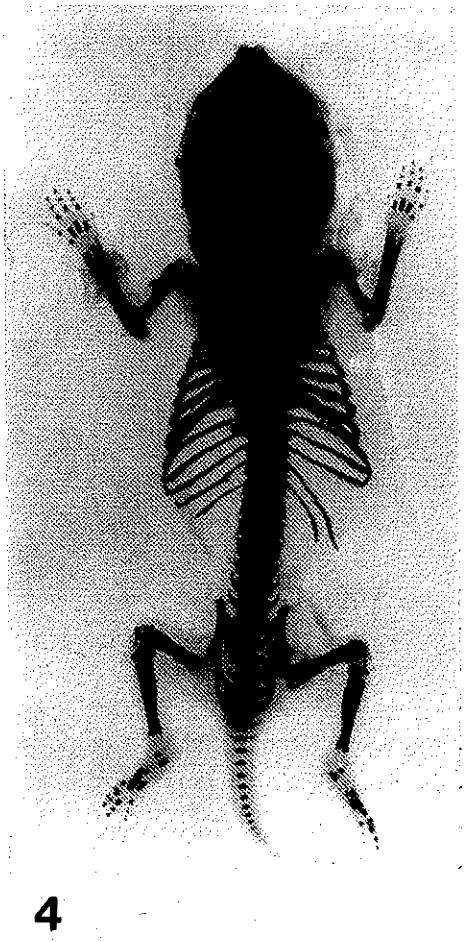


Fig. 4. Skeletal system of a normal newborn mouse. Bone (red) is stained with alizarin red S, and cartilage (blue) is stained with alcian blue.

several studies in both human and experimental animals.[31,47] Kaminski et al.[29] reported lower birth weights in infants born to women who both drank and smoked during pregnancy compared to those whose mothers drank as much but did not smoke. In the study by Sokol and colleages, maternal smoking was found to have an effect additive to that of alcohol.[50] Alcohol alone resulted in a 2.4-fold increase, smoking alone in a 1.8-fold increase, and the combination of maternal alcohol abuse and smoking was associated with a 3.9-fold increase in intrauterine growth retardation.

Recent studies by a Finnish investigator showed a high incidence of limb defects among 453 births to women who both smoked and drank during pregnancy.[2]

MATERIAL AND METHODS

Virgin female A/Jax mice were mated with males of the same strain (3 female: 1 male) overnight and isolated in the morning upon finding a vaginal plug (designated day zero of pregnancy). Mated animals were kept singly in cages at ambient temperature in a room with controlled light and dark periods of 12 hours each.

Mated animals were divided into two groups – controls and experimental animals. The experimental animals were treated as follows:

I. Cigarettes and smoke exposure

High-and low-nicotine cigarettes (NIH code 32, 1.91 mg nicotine and NIH code 13, 0.13 mg nicotine, respectively) were obtained from Oak Ridge National Laboratory, USA.. Nicotine-free cigarettes (Honeyrose, De Luxe, Honeyrose Products Ltd., Stowmarket, Suffolk) were obtained from England. All cigarettes were standard length (85 mm).

Twenty-five pregnant mice were exposed to smoke from one cigarette at 10: 30 a.m. and a second at 11: 30 a.m. on day 10 of gestation. A Walton Smoke Exposure Machine was utilized. The machine was fitted for single cigarette smoke delivery. The smoking protocol was consistent with established procedures for experimental smoke-inhalation studies.

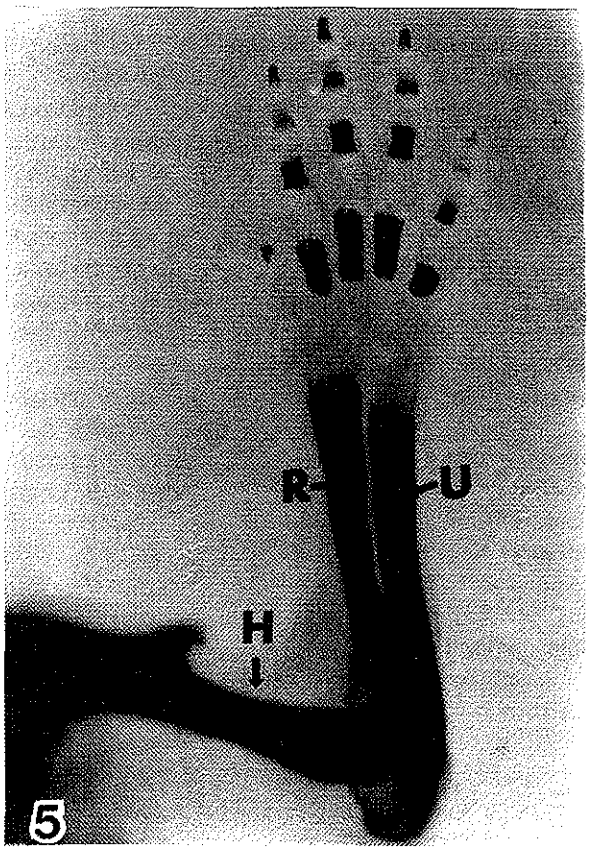


Fig. 5. Forelimb of the control newborn mouse. H = humerus, R = radius, U = ulna.

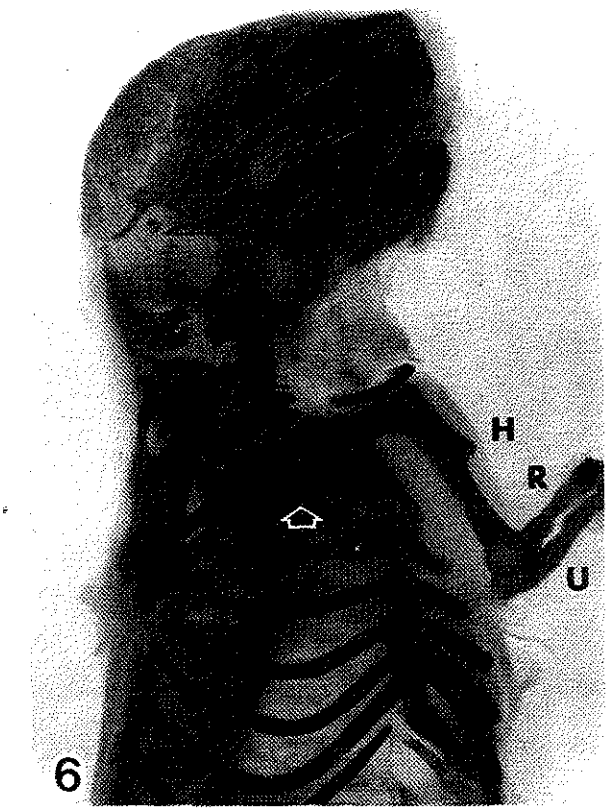


Fig. 6. Experimental newborn mice after clearing and skeletal staining showing ulnar bone and digits on right limb (arrowhead).

II. Ethanol administration

Twenty-five pregnant mice received intraperitoneal injections of 0.015 ml/gm of 25% ethanol both at 8:30 a.m. and at 12: 30 p.m. on day 10 of gestation. Ethanol was diluted in distilled water and administered in a volume of about 1.5 mL. Control animals were injected with an equal volume of sterile saline.

III. Ethanol and cigarette smoke exposure

Twenty-five pregnant mice were exposed to both ethanol (0.015 ml/gm of 25% ethanol at both 8: 30 a.m. and 12: 30 p.m.) and smoke from one cigarette (at both 10: 30 a.m. and 11: 30 a.m.). Control animals for smoking remained untreated.

IV. Detection of skeletal defects

In order to study the teratogenic effects of cigarette smoke, ethanol and ethanol/cigarette smoke on the embryonic skeletal system, the following procedures were conducted: 1) The fetuses as well as newborn animals (both control and treated animals) were fixed in 95% ethanol for one week. The skin and viscera were removed prior to fixation, 2) placed in acetone for 3 days in order to remove the fatty tissue and to keep the specimens firm, 3) placed in freshly prepared staining solution (1: 1 mixture of 0.3% alcian blue 8 GX in 70% ethanol and 1.1% alizarine red S in 95% ethanol) at 37°C for three days, and 4) washed in distilled water, cleared through a graded series of glycerine in KOH for three weeks and stored in 100% glycerine. With this procedure, cartilage is stained blue by the alcian blue and bone stained red with alizarine red S.

V. Scanning electron microscopy

Pregnant animals (both experimental and control) were anesthetized with ether (under a hood) and then killed by cervical dislocation. Embryos (29 experimental and 10 control) with their membranes intact were dissected free of the uterine wall under a dissecting microscope and were placed in 2.5% glutaraldehyde-cacodylate buffer (0.1 M, pH 7.4). Approximately 30 minutes later, membranes were removed and specimens were stored in fixative overnight. All specimens were transferred into cacodylate buffer where they were rinsed and post-fixed in 1% osmium tetroxide in cacodylate buffer for one to two hours. After buffer rinses (3 times), they were dehydrated through a graded alcohol series. The specimens were then dried by the critical point technique, mounted on aluminum studs and sputter coated with gold.

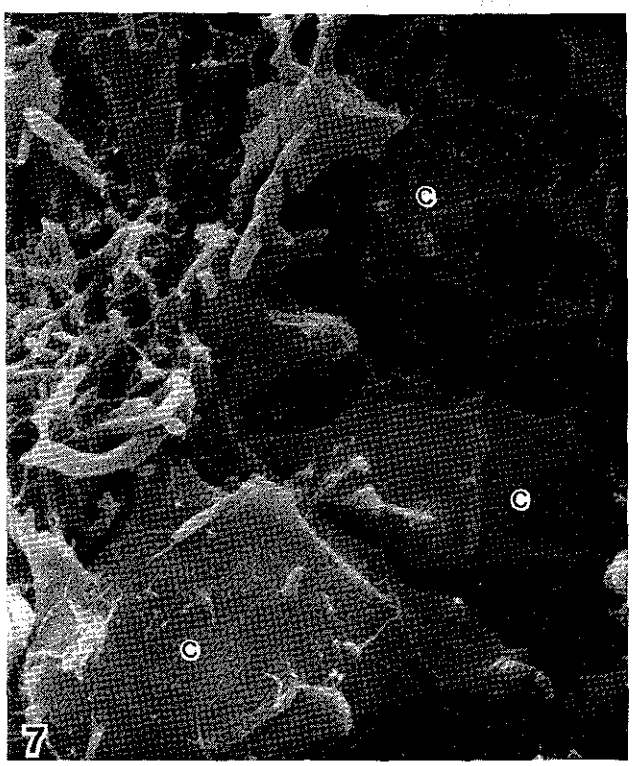


Fig. 7. Mesenchymal cells (c) of limb buds from an embryo whose mother was exposed to both cigarette smoke and ethanol. Note abnormal reduction of cell processes (same magnification and orientation as in Fig. 2).

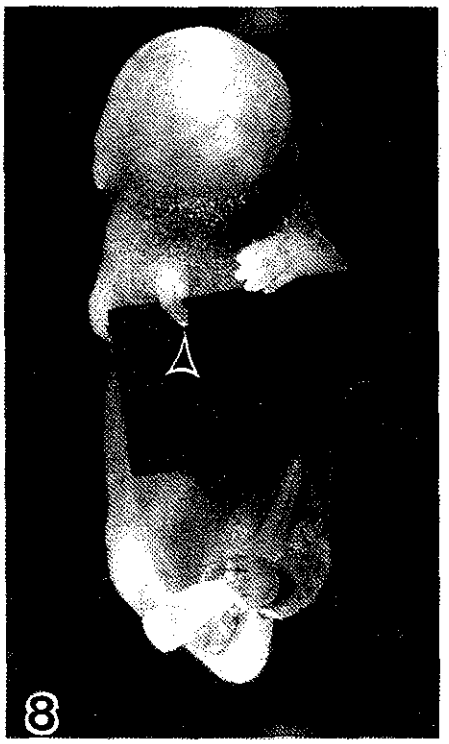


Fig. 8. Experimental newborn mouse showing defects of the right limb (arrowhead) and a shortened left limb.

All samples were examined with an ETEC scanning electron microscope (SEM) operated at 20 kV. Photographic records were made with polaroid type 665 positive/negative film; the negatives were cleared in 12% sodium sulfite. The size and shape of pictures were measured by an SEM scale. Some of the specimens were microdissected after critical point drying, using a hair loop or scotch tape.

RESULTS

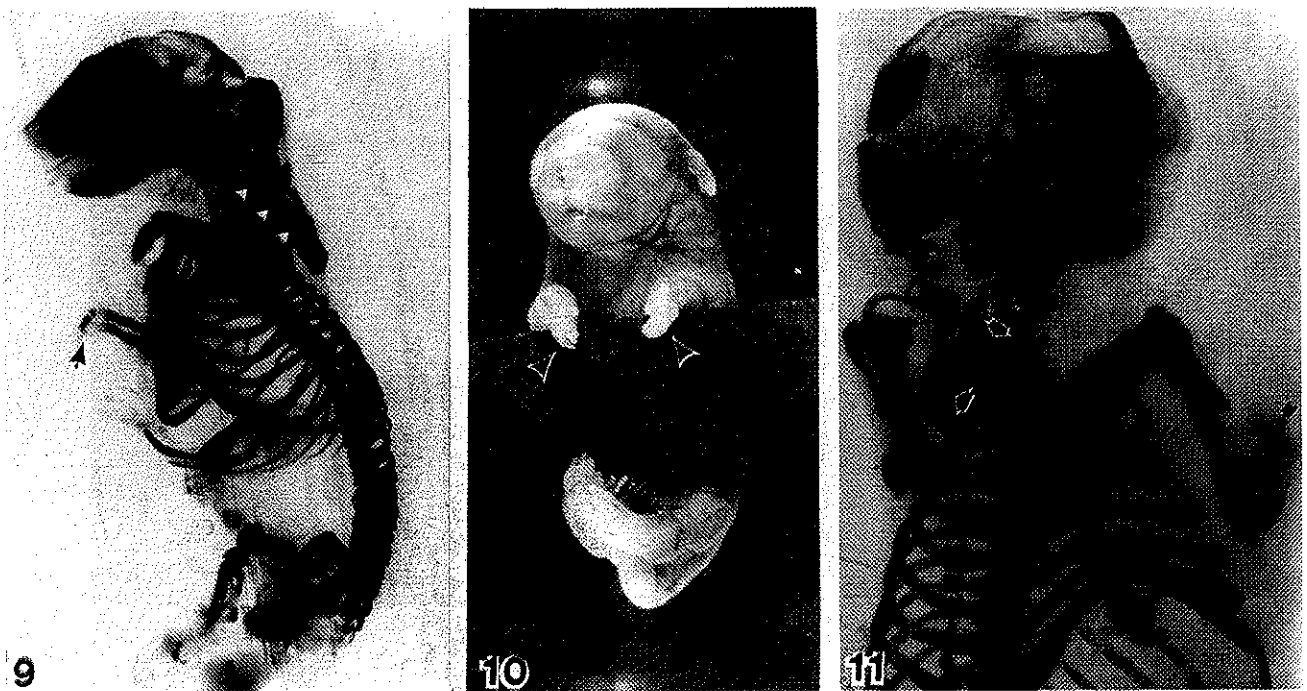
By the time the mouse embryo has about 24 pairs of somites (late day 10 of gestation). the fore limb buds appear at the level of somites 5 to 12; and by the end of day 12 the hindlimb can be observed (Fig.1). SEM studies showed that mesenchymal cells immediately beneath the apical ectodermal ridge, in 11 to 14 day old embryos, exhibited an elaborate network of cell processes (Fig. 2). The basic development of both fore-and hindlimbs is completed by day 16 of development. At birth, all five digits (of each limb) are formed (Figs. 3,4,5).

Of 29 embryos from mothers treated with both ethanol and cigarette smoke (high nicotine), 11 embryos were demonstrably affected. SEM examination of the forelimbs of the affected embryos showed that the surface of the mesenchymal cells near and immediately beneath the apical ectodermal ridge had undergone striking morphological changes. Here, cell processes were either absent or markedly reduced (Fig. 7). Cells at a distance from the apical ectodermal ridge were not affected. Based on the SEM scale, the size and shape of mesenchymal cells from the apical limb region of affected embryos differed drastically from normal (Figs. 2,7).

Of 102 treated newborn animals, 44 had limb defects, and 38 of these also had cardiac defects. In most instances, the postaxial (ulnar) side of the forearm was affected, and the ulnar bone and all digits were missing (Figs. 6,10).

Most affected newborns had bilateral limb defects similar to those shown in Figs. 10 and 11. In a few cases, in addition to postaxial limb defects, the forearm (both the radius and ulna) was shorter than in normal newborns (compare Figs. 8,11 with Figs. 3,4; and Figs. 8,9,11 with Figs. 3,4,5). The unilateral defects were mostly on the right side of the newborn animals (Fig. 8). The limb defects seen here did not occur in the offspring of mothers exposed to either of these two teratogens alone. Offspring of mothers exposed to smoke from low-nicotine or nicotine-free cigarettes did not develop any limb defects.

The length of gestation in control animals was 20 days. In cigarette smoke and/or ethanol-treated animals, the gestational period increased to between 22 days, and the



Figs. 9, 10, 11. Experimental newborn mouse with bilateral forelimb defects (arrowheads).

average weight of newborns was less than normal newborns.

DISCUSSION

One of the prime goals of experimental teratology is to clarify the mechanism (s) involved in teratogenesis. For this purpose, it is necessary to find a suitable teratogen and a sensitive species of animal that produce a high incidence of a certain type of malformation. Teratogens, animal models and techniques used in this study provide us with a substantial amount of morphological information and set the stage for a more detailed analysis of the genesis of specific defects at the molecular level. This research has also generated data concerning the optimal dosage of teratogen and time of administration needed to produce a high incidence of defects in developing mice embryos. Due to the fact that there is a high level of spontaneous malformations[24,25,56] among A/Jax mice, a large number of controls were studied in order to establish a reliable baseline percentage of specific defects.

In the experiments described here, we found that the morning of day 10 of gestation (3-4 hours prior to the 1-3 tail somite stage) is an optimum time for experimental production of limb defects. This emphasizes the fact that there are "critical stages" in the development of limbs in which teratogens may exert their effects. It is well known that effects of insults during critical periods of development are usually irreversible.[9,10,32]

We were able to produce a high incidence of limb defects co-existing with cardiac malformations by treatment of pregnant mice with a combination of ethanol and smoke from high-nicotine cigarettes (The cardiac defects are not presented in this paper but will be submitted for publication soon). In most cases the post-axial (ulnar) side and the distal ends of forelimbs were affected. The ulnar bone, as well as most digits, were not formed in affected embryos. Cellular studies showed that, subsequent to maternal treatment, the mesenchymal cells immediately beneath the apical ectodermal ridge undergo striking morphological changes (Fig. 7). In control embryos, mesenchymal cells exhibited an elaborate network of cell processes. This network was much reduced in treated embryos. We postulate that the changes in these mesenchymal cells are related to the later appearance of gross limb defects. Perhaps the changed morphology of these cells reflects a failure of normal tissue interactions between the apical ectodermal ridge and its underlying mesenchyme. Because it has been shown that such interactions are very important for further development of the limbs, its disruption by teratogens could result in limb defects among newborns.[22,60] These morphological changes might be due to direct or indirect effects on cytoskeletal elements (e.g., the tubulin assembly). Therefore, it would be useful to determine whether the distribution of tubulin, actin, etc. is altered by teratogen treatment.

Smoke from high-nicotine cigarettes alone did not produce any kind of limb defect. Administration of ethanol alone produced a low percentage of forelimb defects (1.2%). The teratogenic interaction between drugs has been reported by many investigators.[31,45,51] Of particular interest is a report by Aro[2] that shows congenital limb malformation among

the offspring of Finnish women who were both heavy smokers and drinkers. Smoking can cause hypoxia in the fetus.[18,38] It is possible that the combination of ethanol and nicotine and/or their metabolites under hypoxic conditions, and at critical stages of development for both heart and limb can cause these combined defects in experimental animals (or in humans). Because ethanol alone produced a very low percentage of forelimb defects, other agents (nicotine, its metabolites, hypoxia) may have a potentiative interaction with ethanol and enhance its effects.

An alteration of the extracellular matrix by teratogen might also cause developmental defects. Components of this matrix may serve to control cell-cell or tissue-tissue interactions, e.g., those occurring between the apical ectodermal ridge and the underlying mesenchyme in limb buds. In addition to tissue interactions, the regional compositional differences in the extracellular matrix (such as collagen, glycosaminoglycans, etc.) may also provide the stimulus for other developmental phenomena such as proliferation and migration as well as controlling morphogenetic movements.[3,11,21,22,44,53,63] Analysis of these components by means of other techniques (immunocytochemical, biochemical, etc.) before and after exposure of mothers to teratogenic insults might be very useful in understanding not only the mechanism of teratogen action but also the normal events occurring during critical developmental periods.

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