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Acquired Chromosomal Anomalies Induced in Mice by Injection of a Teratogen in Pregnancy

The experiments herein reported were undertaken to test whether a selected teratogen that can cause anomalies in 95 percent of fetuses can also cause chromosomal anomalies. 6-Amino nicotinamide was selected as the teratogenic agent, cleft palate as the deformity to be induced, and pregnant outbred females of the 15-year-old Phipps mouse colony as the biologic model to be used in the experiments. This agent, this defect, and this animal were chosen because much work had been done on chemical, morphologic, and embryological aspects of all three variables (*1*).

Pilot studies were made to establish the critical period for production of cleft palate by 6-amino nicotinamide in offspring of pregnant females of the Phipps mouse colony. Standard methods were modified and applied for the demonstration of chromosomal structure of cells of tissues adjacent to and remote from palatal defects induced by the administration of 6-amino nicotinamide to pregnant females around the 13th day of gestation. Chromosomal analyses were also carried out on maternal bone marrow, a tissue regularly in a high state of mitotic activity, to determine whether or not 6-amino nicotinamide injection had any pathogenic effect on maternal chromosomal patterns. For purposes of comparison similar studies were made of chromosomal patterns in cells of palates and other tissues from normal embryos of untreated mothers. Search was also made for presence or absence of chromosomal anomalies in cells from bone marrow of mothers of control embryos.

The following steps, with modifications of standard techniques (*2*, *3*), formed the basis of procedure. Some pregnant animals were killed by ether inhalation 1 day after injection of 6-amino nicotinamide; most were killed 6 days after injection or as controls on the 19th day of gestation. Under sterile conditions, embryos were taken from the uterus and placed in a sterile petri dish with a few milliliters of nutrient (medium 199 containing 15-percent calf serum). The embryos were examined at this time to ascertain the presence or absence of cleft palate. The embryonic material was then processed for observation of cellular structure and chromosomal patterns of cells arrested in metaphase. Some pieces of palatal tissue, muscle, skin, and limb fragments were removed and processed separately as plasma clot preparations on cover slips in stationary roller tubes or Leighton tubes according to a method previously described by Ingenito and her colleagues (*2*). The remaining part of the embryo was then trypsinized, and the resulting cell suspension planted in similar culture tubes containing cover slips. In the early outgrowths—about 18 hours after explantation of trypsinized material, and about 36 hours after explantation for plasma clot preparations—the cells consist of two distinct types. One type is round and granular; the other is an elongated fibroblast-like cell with long processes. Within 1 to 2 days, the round cells show only a slight increase in number, whereas the fibroblast-like cells grow more rapidly out into a monolayer. At this time of most rapid proliferation, the cultures have reached their peak of mitotic activity, and the outgrowth on the cover slip is ready to be fixed and stained for chromosomal analysis.

The normal chromosomal pattern of cells cultured from the mouse consists of 40 similar-appearing chromosomes, with variations of 39 or 41 chromosomes occasionally encountered. One to 2 percent of these mitotic cells may exhibit polyploidy, and 3 to 4 percent may show fragmentation (Fig. 1 and Table 1).

When 6-amino nicotinamide is administered to pregnant females around the 13th day of pregnancy, about 95 percent of the embryos subsequently manifest cleft palate at section. The outgrowths from tissues of these embryos are composed of a loose meshwork of large vacuolated cells with coarse cytoplasmic granules and pyknotic nuclei (Fig. 2).

Microscopic study of chromosomal structure of these cells reveals that chromosomal anomalies have been produced by the teratogen used (Fig. 1), not only in cells grown from tissues adjacent to palatal defects, but also in cells grown from tissues in areas remote from the palate. Many more cells exhibiting polyploidy and fragmented chromosomes are observed in the offspring of test animals than in the offspring of normal controls. These findings are shown quantitatively in Table 1, the data of which also give clear indication that a time factor is involved. Polyploidy, for example, characterized almost half the mitotic cells examined within 24 hours after the injection of the teratogen; 9 percent of mitotic cells examined 6 days after injection; and in only about 2 percent of mitotic cells taken from control embryos. Not only were chromosomal anomalies found in cells cultured from embryonic tissues but the same kind of anomalies were demonstrated on examination of processed bone marrow of injected mothers 6 days after the administration of the teratogen (see Table 2).

Although the chemical explanation for the action of 6-amino nicotinamide in causing the chromosomal anomalies described is unknown, the epidemiologic (time-place-person) events in pregnancy are known. Had these events not been observed, the discovery of

Table 1. Normal and abnormal chromosomal patterns of mouse embryos 1 day and 6 days after injection of 6-amino nicotinamide.

Days after injection	Cells counted in metaphase	Normal pattern		Polyploidy		Fragmentation	
		No.	%	No.	%	No.	%
1	204	85	41.7	97	47.5	22	10.8
6	395	242	61.3	36	9.1	117	29.6
Control embryos	219	207	94.5	4	1.8	8	3.6

chromosomal anomalies in the maternal bone marrow associated with chromosomal anomalies and congenital malformations of the fetus could easily have been misinterpreted as evidence of a genetic defect passed on from mother to progeny.

In view of this, perhaps the most useful application of the work reported here is to serve as a model to help shape investigations of the numerous factors that need to be considered and redefined in experimental studies undertaken at population levels. In mice both intrinsic host factors and extrinsic environmental circumstantials involved in the determination of chromosomal anomalies can be isolated, quantitated, and studied experimentally. In particular, further studies are indicated to test whether the palatal defect itself is an expression of chromosomal aberration or whether the congenital deformity is the direct result of injury to primordial tissue and whole clones of cells in the embryonic palate. That there needs to be critical timing of a teratogenic stress in order to produce experimentally a selected anomaly in mammals—for example, cleft palate in mice—was demonstrated by Ingalls, Curley, and Prindle (*4*) in 1950. That timing of an environmental stress is also a critical factor in the production of chromosomal anomalies is implicit in the high frequency of polyploidy observed 1 day after injection of the teratogen compared with the frequency of polyploidy in mitotic cells examined 6 days after the injection of 6-amino nicotinamide.

The findings point to an emerging experimental epidemiology of genetic anomalies which can be related to the experimental epidemiology of congenital malformations and monstrosities that has already developed in the past 30 years. In these animals, fish, birds, mice, rats, rabbits, and sheep, profound abnormalities of the fetus may be induced by injury of the very early embryo. Yet to be determined is the degree to which destruction of chromosomal substances, pluripotential, primordial cells, or clones of differentiating cells contribute to final results. Since genetic substance itself can be shown to be susceptible to environmental injury, the hypothesis is advanced that, once a chromosomal anomaly is induced, the impact may be far-reaching for the subsequent generations of cells to be descended from the injured individual. The findings so far go a

Table 2. Normal and abnormal chromosomal patterns of processed maternal marrow cells 6 days after injection of 6-amino nicotinamide.

Source of cellular outgrowth	Number observed	Normal pattern		Polyploidy		Fragmentation	
		No.	%	No.	%	No.	%
Controls	159	137	86.2	6	3.8	16	10.1
Mothers of cleft palate embryos	84	30	35.7	5	6.0	49	58.3

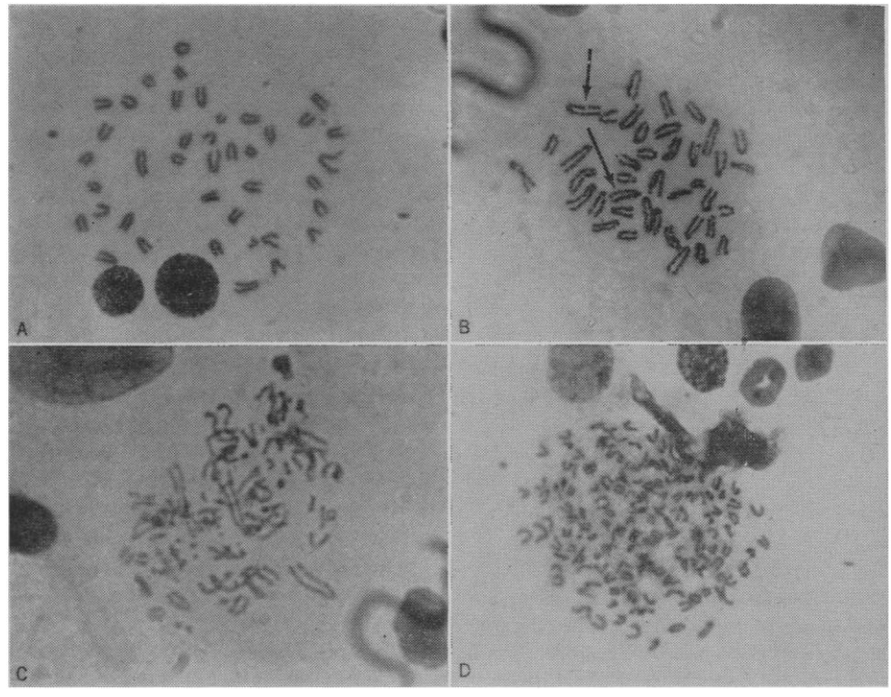

Fig. 1. Chromosomal anomalies in mouse embryos after maternal injections of 6-amino nicotinamide on the 13th day of pregnancy. *A*, Normal diploid cell with 40 telocentric chromosomes; *B*, cell with fragmentation of chromosomes; *C*, cell with extensive fragmentation of chromosomes; *D*, polyploid cell.

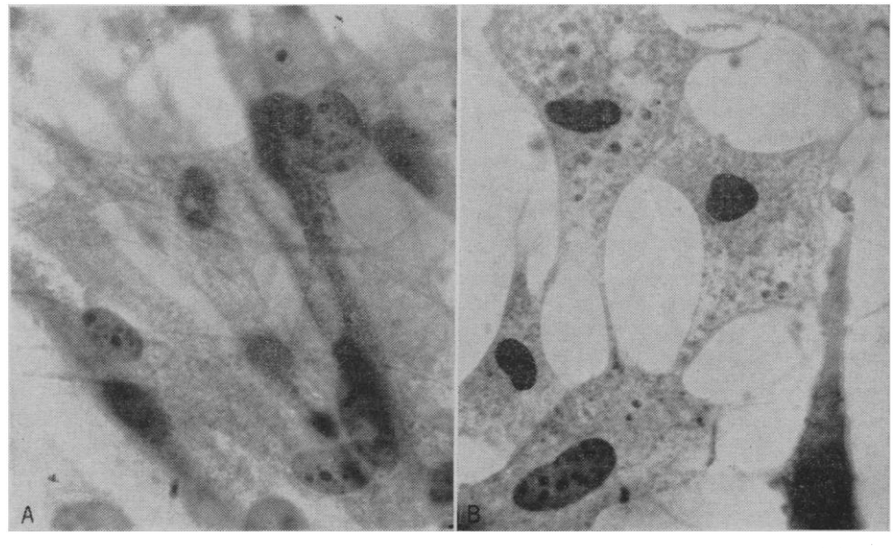

Fig. 2. Cellular outgrowths from tissue cultures of embryonic mouse material. *A*, Normal fibroblast-like cells from control material 48 hours after explantation; *B*, 48-hour outgrowth from test material after maternal injections of 6-amino nicotinamide on the 13th day of gestation.

long way toward harmonizing many discoveries of the developmental geneticist (focusing to a greater extent on innate endowment) with those of the experimental zoologist (focusing more largely on environmental adversity) in respect to causes of congenital malformations.

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2 July 1963

Fixation of Complement to Fragments of Antibody

Abstract. *A specific precipitate of ovalbumin and its rabbit-serum antibody, after fixing human serum complement, was digested with papain. The digest was analyzed immunochemically for complexes of antigen, antibody fragments, and components of complement. The results indicated that complement is not bound to Porter fragment III, but very likely is bound to fragments I and II.*

The amount of complement (C′) fixed by antibody-antigen complexes at or near equivalence is proportional to the antibody present. Some antibodies, however, fix complement poorly or not at all despite their precipitation by homologous antigen. Precipitation of antibody by antigen is therefore not a sufficient condition for antibody to fix complement, and it has been assumed that fixation depends also upon other structural characteristics of antibody. It became possible to obtain direct evidence relating antibody structure to complement fixation when Porter (*1*) showed that antibody digested with papain-cysteine yields three distinct fragments, and Nisonoff *et al.* (*2*) obtained by pepsin digestion two pieces, one of which consisted of Porter fragments I and II.

Taranta and Franklin (*3*), Amiraian and Leikhim (*4*), and Ishizaka *et al.* (*5*) examined the Porter and Nisonoff fragments for their capacity to fix C′ in the presence of antigen or to inhibit immune lysis of sensitized sheep erythrocytes and concluded that fragment III is the portion of the antibody molecule associated with complement fixation.

Our approach to the problem of determining which of the antibody fragments is required for complement fixation differs from theirs in that complement was fixed to an antibody-antigen system under normal conditions before digestion of antibody by papain-cysteine. The specific precipitate, having fixed complement, was digested and examined for complexes consisting of antibody fragments, antigen, and components of complement which might have remained bound. Results of experiments of this type led to the conclusion that complement is not bound to fragment III but is probably fixed to fragments I and II of the antibody molecule.

In our experiments we used ovalbumin and its rabbit-serum antibody as the antigen-antibody system, fresh human serum as the source of complement, and heat-inactivated human serum (56°C, 30 min) as control. Components of complement from heat-inactivated serum are not taken up by immune-specific precipitates (*6*). For this reason the rabbit antiserum was also heated at 56°C for 30 minutes. The order in which the reactants were mixed was antiserum, human serum (C′) or saline, and antigen. Each mixture contained 12.5 mg of antibody nitrogen, enough antigen to precipitate the antibody at equivalence, and 100 ml of fresh human serum, heat-inactivated serum, or saline. After 4 hours at 37°C and about 16 hours at 0°C, the specific precipitates were separated, washed thoroughly until the supernatants were free from inert constituents of rabbit antiserum and human serum, and digested with mercuripapain in the presence of cysteine and EDTA. The soluble papain digest was dialyzed against 0.01*M* acetate buffer (*p*H 5) and centrifuged. The water-soluble fraction, upon chromatography on carboxymethyl (CM) cellulose, yielded three fractions.

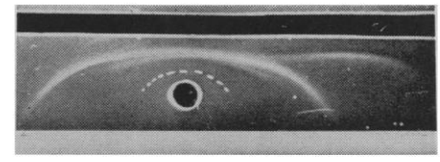

Fig. 1. Immunoelectrophoretic analysis of a saline-soluble extract for antibody fragments. The extract was from a specific precipitate containing C′. Sheep antirabbit serum was added to the trough. Electrophoretic migration was to the right toward the anode.

The insoluble fraction was taken up in 0.15*M* saline-veronal buffer (*p*H 7.4) giving the saline-soluble fraction. The original digest and its fractions were analyzed by gel-diffusion methods for antigens derived from rabbit anti-ovalbumin, ovalbumin, and human serum. Rabbit antisera to human serum were used for the detection of constituents of human serum. One was a polyvalent antiserum and the other was prepared against the fraction of human serum which does not contain γ-globulin. Both inactivated specifically the hemolytic activity of human serum and therefore contained antibodies against hemolytic components of complement. Sheep antirabbit serum was used to detect fragments of rabbit anti-ovalbumin. The digest was also tested for precipitating antibody against ovalbumin; none was found, indicating that the digestion of rabbit antibody had been complete.

The chromatographic patterns of the water-soluble fractions were identical whether or not the water-soluble fractions were derived from specific precipitates containing C′; three fractions were recovered from each. Fractions

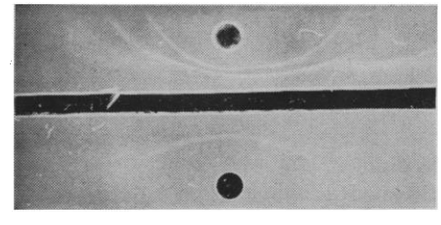

Fig. 2. Immunoelectrophoretic analysis for components of C′. The top well contained the same saline-soluble fraction used in the analysis in Fig. 1. The bottom well contained the water-soluble fraction of the digested specific precipitate formed in the presence of C′ from fresh human serum. Rabbit antihuman serum was added to the trough. Electrophoretic migration was to the right toward the anode.