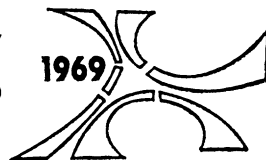


Leukocyte Chromosome Study of 22 Families with Cleft Lip and/or Cleft Palate Members



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Oral facial clefts are a heterogeneous group of anomalies that can be attributed to various hereditary and nonhereditary factors. For example, patients with autosomal trisomy syndromes such as Down's, $\#13-15$ (D_1) trisomy and $\#18$ trisomy syndromes, have cleft palate among many other malformations (18). Cleft lip with or without cleft palate ($CL \pm CP$), or cleft palate alone (CP) may also be present in patients who have some rare inherited diseases, such as *fistula labii inferioris congenita*, Marfan's syndrome, and mandibulo-facial dysostosis (4). However, the overwhelming majority of the clefts do not seem to be associated either with gross chromosomal anomalies or with a demonstrable simple mode of inheritance.

Chromosome studies on the cleft patients have been reported from several laboratories. Two sources are generally used for chromosome analysis. One is the peripheral leukocytes; the other is the palatal tissue taken from cleft patients during corrective surgery.

Leukocyte chromosomes have been studied in 6 cases by Makino (8) and in 11 cases by Subrt and associates (16). The karyotypes were found to be normal in all these cases but one. The only exception was a male patient with Down's syndrome among those studied by the latter group of the investigators.

Palate tissues have been studied in 8 cases by Jackson (6), 6 cases by Soukup and Warkany (14), with negative finding as regards abnormal karyotypes. However, epithelial-like cells from the palate tissue of a boy patient were reported by Gropp and associates (5) as having a near-triploid chromosome number. Murphy and Reisman (11) also reported 2 cases (out of 10) of palatal cultures having excess proportions of aneuploid cells. Thus, of these 16 patients (excluding the case of

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Down's Syndrome) studied with leukocyte method and 25 patients with the tissue method, one was shown to have near-triploid chromosome number; three were shown to have high proportions of aneuploid cells. These results indicate that only a small portion of cleft patients have detectable abnormal chromosome features.

However, in view of the higher incidence of clefts among the relatives of cleft patients as compared to the population in general (12, 15, 19), one approach to the relationship of clefts and chromosome anomaly will be to study the chromosomes of various members of families, which have at least one such cleft patient, and to see whether anything discernible can be detected by the current chromosome methodology. Due obviously to the very limited access to source materials, the chromosome studies of cleft families have not thus far been reported. In the following, a chromosome study of the members of 22 cleft families is reported.

Materials and Methods

Because of the extensive clinical program at the Lancaster Cleft Palate Clinic, we had ready access to patients and their family members, who willingly gave their blood for leukocyte cultures. These families, totaling 22, were from Lancaster County and its five adjacent counties in Pennsylvania. Blood samples were obtained over a period of two years from these persons as they came to the Clinic for their scheduled visits. The members of these families ranged in ages from 2 to 59. Blood samples were also taken from a group of 10 unrelated individuals (4 weeks to 40 years old) whose immediate families have no known cases of clefts.

BLOOD MICROCULTURE. The microculture method used in this study was as follows. A few drops of blood, obtained by a finger prick, were collected in a sterile tube containing 0.2 ml heparin (Abbott Labs, 1,000 units/ml), and were then transferred to culture tubes which contained 4 ml Eagle's basal medium (Grand Island Biological Co., Grand Island, N. Y.), 1 ml fetal calf serum, 0.2 ml bean extract,¹ and 1,000 units penicillin and 1,000 μ g streptomycin. All cultures were incubated at 37°C. Colcemid (1.6×10^{-5} mg/ml final concentration) was added at 68 h and the cultures were harvested 4 h later. After hypotonic treatment in 0.7% sodium citrate for 10 min at 37°C and centrifugation, the cells were immediately suspended in a fixative of 3:1 absolute methanol-glacial acetic acid. The suspension was centrifuged, and the fixative was replaced by a fresh aliquot. After repeating twice the fixative washing procedure, the cells were suspended in a small amount of fresh fixative

¹ Soak beans in physiological saline (1 gm bean:2 ml saline); homogenize, centrifuge the suspension, and keep the top layer; discard the rest. Dilute the supernatant 1:5 with sterile Hank's balanced salt solution and store frozen. For working solution, dilute further 1:10 with Hank's BSS and use 0.2 ml of the working solution in each culture tube.

to give a proper dilution. Slides, prepared by an ignition-drying method (9), were stained for 6 min in 10% Giemsa stain (with 10% 0.15N ammonium hydroxide), washed twice in acetone, once in 1:1 (v/v) acetone-xylene, twice in xylene, and mounted.

CHROMOSOME ANALYSIS. After the selection of adequately spread metaphases was made with the aid of a 10X objective, chromosomes were counted and the karyotype analyzed for the presence of any abnormality (achromatic gaps, true breaks, rings, dicentrics, et cetera) by using an oil immersion lens. Achromatic chromosome gaps, whether they were wide or narrow trans-chromatid spaces, were scored as definite gaps only if they appeared well defined and without visible connecting strands of chromatin. Secondary constrictions identified in the human chromosome complement were not scored as achromatic gaps even if their appearance met the above criteria. Achromatic gaps were scored as true breaks if there were positional displacements of chromatids. Whenever possible, 50 metaphases were analyzed from each blood culture.

Results

In all, 97 individuals from 22 families were studied. Neither numerically abnormal karyotype nor gross chromosomal structural rearrangements (such as dicentrics, rings, and translocations) were found in these subjects, except in one cell from a girl sibling. The cell in question had one exchange figure involving two $\times 13-15$ chromosomes. Two hundred additional cells from the same girl were analyzed, but no further structurally abnormal chromosome was found. In 4 of 6 members of another family, a $\times 17-18$ chromosome with pronounced satellites in the short arm was observed in the cleft as well as in the normal members of that particular family.

Since it is known that $CL \pm CP$ is etiologically different from CP (3, 4, 12, 15, 19), the results presented in Tables 1 and 2 are grouped into two broad categories: "A" families, in which the affected siblings had $CL \pm CP$; and "B" families in which the affected siblings had CP only. Further, since we studied the *propositi* and their immediate family members (that is, father, mother, sisters and brothers), each family group was divided into three subgroups: a) parents, b) normal siblings of the *propositi*, and c) *propositi* and their siblings who also had clefts (usually the same type of cleft as those of *propositi*). There is one important difference between the two tables however. In Table 1, all parents were normal, whereas in Table 2, at least one parent of each family had a cleft. In each table, the percentages of cells having true breaks and/or achromatic gaps and the percentages of cells having true breaks (disregarding the achromatic gaps) are presented.

It is difficult to decide which statistical test to apply to the data in order to determine whether or not the percentages of chromosome breaks

TABLE 1. Chromosome analysis of families with noneleft parents. "A" families, having probands with cleft lip $\pm$ cleft palate; and "B" families, having probands with cleft palate only.

	<i>"A" families</i>				<i>"B" families</i>				
	<i>parents</i>	<i>sibs, non-cleft</i>	<i>sibs, cleft</i>	<i>total</i>	<i>parents</i>	<i>sibs, non-cleft</i>	<i>sibs, cleft</i>	<i>total</i>	<i>unrelated subjects (normal)</i>
number of individuals	18	16	10	44	9	5	11	25	10
cells analyzed	787	784	484	2055	451	250	504	1205	479
cells with breaks, gaps	117	124	96	337	63	41	80	184	88
(%)	(14.8)	(15.8)	(19.8)	(16.3)	(13.9)	(16.4)	(15.8)	(15.2)	(18.3)
cells with true breaks	20	14	10	44	5	1	6	12	3
(%)	(2.5)	(1.7)	(2.0)	(2.1)	(1.1)	(0.4)	(1.2)	(0.9)	(0.6)

TABLE 2. Chromosome analysis of families. "A" families, with parents and probands having cleft lip $\pm$ cleft palate; and "B" families, with parents and probands having cleft palate only.

	<i>"A" families</i>				<i>"B" families</i>				
	<i>parents</i>	<i>sibs, non-cleft</i>	<i>sibs, cleft</i>	<i>total</i>	<i>parents</i>	<i>sibs, non-cleft</i>	<i>sibs, cleft</i>	<i>total</i>	<i>unrelated subjects (normal)</i>
no. individuals	7	4	5	16	5	3	4	12	10
cells analyzed	338	165	233	736	201	129	154	484	479
cells with breaks, gaps	64	32	50	146	21	3	26	50	88
(%)	(18.9)	(19.3)	(21.4)	(19.8)	(10.4)	(2.3)	(16.8)	(10.3)	(18.8)
cells with true breaks	10	2	9	21	4	1	4	9	3
(%)	(2.9)	(1.2)	(3.8)	(2.8)	(1.9)	(0.8)	(2.5)	(1.8)	(0.6)

and/or gaps in various subgroups are significantly different from each other. The nonparametric methods (10) can be used without regard to the form of population (normal or binomial) from which these samples were taken. However, in the following, a binomial distribution is assumed. The question asked is: Can the subgroups in each family group be regarded as the different samples from the same population? Consequently, a test of significance for the binomial population was performed for every possible combination of two subgroups (1 vs. 2, 1 vs. 3, 2 vs. 3) according to the formula given by Li (7).

In Table 1, within either category of families, the percentage of cells

with breaks and/or gaps are similar in various subgroups, with one exception. The exception is that the percentage of breaks and/or gaps in parents (of "A" families) is significantly lower than in cleft-sibs at 5% level of significance. If all the achromatic gaps are disregarded, there are no differences among the frequencies of cells with true breaks within either family group. If we combine the results in each family group, we obtain 16.3% for breaks and/or gaps in "A" families, and a corresponding value of 15.2% for "B" families. The difference between the above two figures is not significant at 5% level of significance. Both figures are, incidentally, *not* significantly different from the control (18.3%). However, if we compare the combined frequency of breaks alone in "A" and "B" family groups, the difference (2.1% vs. 0.9%) is significant at 5% level. Likewise, the combined break frequency (2.1%) in "A" is significantly higher than that of unrelated normal subjects (0.6%), although the combined frequency of breaks in "B" (0.9%) is not.

The same trend also prevails for the data presented in Table 2. In "A" families, there is no significant difference in the frequencies of cells with breaks and/or gaps among the three groups; their combined frequency amounted to 19.8%, which is not different from the control (18.3%). In "B" families, however, the corresponding frequencies for breaks and/or gaps are lower than those of "A" families; and the combined frequency of 10.3% in "B" families is significantly lower than both "A" families (19.8%) and the control (18.3%).

As for the true break frequencies in Table 2, there is no significant difference among groups in either "A" or "B" families. The combined break frequency of "A" families (2.8%) is significantly higher than the unrelated control (0.6%), while the corresponding difference between "B" families and unrelated control group (1.8% vs. 0.6%) is not significant.

In short, our data tended to show that the family members from the "A" family group as a whole had a higher frequency of *true breaks* than those of unrelated persons. On the other hand, the data at hand showed no such significant difference between the members of the "B" family group and the unrelated controls. When the frequencies of breaks and/or gaps are considered, there are no such consistent results.

Since it is well established that the relatives (especially the siblings, parents, and children) of propositi with $CL \pm CP$ and CP have a higher incidence of clefts similar to the cleft types of the propositi (3, 4, 12, 15, 19) Tables 1 and 2 were combined and presented in Table 3. The conclusions reached for Tables 1 and 2 also apply to Table 3: while there were some variations in the frequencies of cells with breaks and/or gaps, the combined frequency of true breaks in "A" families was significantly higher than either in the unrelated group of individuals or

TABLE 3. Chromosome analysis of families having cleft lip $\pm$ cleft palate or cleft palate children.

	"A" families				"B" families				
	parents	sibs, non- cleft	sibs, cleft	total	parents	sibs, non- cleft	sibs, cleft	total	unre- lated sub- jects (nor- mal)
no. individ- uals	25	20	15	60	14	8	15	37	10
cells ana- lyzed	1125	949	717	2791	652	379	658	1689	479
cells with breaks, gaps	181	156	146	483	84	44	106	234	88
(%)	(16.08)	(16.43)	(20.22)	(17.3)	(12.88)	(11.60)	(16.10)	(13.85)	(18.3)
cells with true breaks	30	16	19	65	9	2	10	21	3
(%)	(2.66)	(1.68)	(2.64)	(2.33)	(1.38)	(0.52)	(1.51)	(1.24)	(0.6)

in the "B" families. The frequency of breaks in "B" families was not significantly higher than the control.

Discussion

Our results from this family study showed that the members of the families with CL $\pm$ CP as a whole had more chromosome breaks in their leukocytes than did the members of the families which had CP only, or the unrelated control group. If the finding can be confirmed, it will be important to ask why this should be so.

According to Canick (2), the human lip development takes place during the 5th to 8th week of gestation, whereas the palatal region develops during the 9th week. We also have ample evidence to suggest that CL $\pm$ CP and CP are etiologically different entities. CL $\pm$ CP in addition has "a large hereditary element" (12), in contrast to CP for which no such large genetic component has been established. In view of the above, the results of our present study is understandable.

What is responsible for the presence of more chromosome breaks in the leukocyte cultures from CL $\pm$ CP patients? We do not know. But, some of the factors involved in the induction of chromosome breaks are known.

First of all, *genetic factors* may cause high frequency of chromosome breaks. Thus, it has been reported that the cells from Fanconi's anemia patient shows increased chromosome breaks (17). Other exogenous factors such as X rays, alkylating agents, anti-metabolites, drugs and chemicals (streptonigrin, mitomycin C, LSD-25, et cetera) induce chro-

mosome breaks. With so many chemicals capable of inducing chromosome breaks, it is possible that different agents produce chromosome breaks through different modes of action. Although we have good documentation of the chromosome-breaking agents, we still don't know exactly how these agents produce chromosome breaks. However, it has been suggested that subnormal ATP and protein syntheses are among those factors which can explain the induction of chromosome breaks.

ATP is a principal source of energy for many biological synthetic activities. Deprivation of ATP, which provides the energy for repair processes, potentiates the x-ray damage (including chromosome breaks) in cells and organisms. Furthermore, ATP is of great interest to teratologists because many uncoupling agents of oxidative phosphorylation have been known to be teratogenic. Very recently, it has been reported that tissues from rats fed riboflavin-deficient diet had less activities of the terminal electron transport system than the normal tissues (1). Perhaps it is no coincidence that some sort of damage or inhibition in the ATP-generating system has been implicated in radiation biology, chromosome breakage, and teratology. ATP seems to be the common denominator in them. Is the ATP-generating system defective in the CL $\pm$ CP tissues? We don't know. It is then reasonable to anticipate that studies of the oxidative phosphorylation of the cleft tissues may prove to be a very important and profitable facet of the studies of CL $\pm$ CP patients.

In connection with the discussion of ATP in cells, it should be mentioned that the source of energy in the phytohemagglutinin (PHA)-stimulated leukocyte cultures is glycolysis. If the glycolysis is inhibited, for example, by addition of 10 mM 2-deoxy-D-glucose (which inhibits the phosphohexoisomerase), the syntheses of DNA, RNA and protein are inhibited also (13). Similarly, our preliminary experiments showed that the treatment of PHA-stimulated leukocyte cultures with 10 mM 2-deoxy-D-glucose for more than 8 hours reduced the mitosis in the cultures to nil. It should be interesting to see if appropriate conditions of 2-deoxy-D-glucose treatment could be found which will allow some degree of mitosis and yet produce chromosome breaks in the leukocyte cultures. If proper treatments can produce chromosome breaks in the leukocyte cultures, it will be pertinent to ask: Is the glycolysis in the leukocytes from CL $\pm$ CP patients impaired so that this is reflected in the higher frequency of true chromosome breaks? This is one of the many questions awaiting answer from future investigations.

Summary

Micro-cultures of leukocytes (obtained by finger-tip puncture) were established from 97 members of 22 families each having at least one member either with CL $\pm$ CP or CP. Subjects ranged in ages from 2 to 59. At the same time cultures were also established from 10 unrelated individuals (4 weeks to 40 years old) from normal families. Results

showed no abnormal karyotypes and no detectable chromosomal structural rearrangements. However, the data on the break frequency of chromosomes in the cultures showed that a significantly higher frequency (2.3%) of breaks occurred in those families whose affected members had cleft lip $\pm$ cleft palate, as opposed to the control group of unrelated individuals (0.6%), or the group of families whose affected members had cleft palate only (1.2%). Interpretation of the finding about breakage frequencies is difficult. Genetic factors and external agents such as X rays, alkylating agents, drugs and chemicals are known to cause chromosome breaks. The mechanisms of chromosome breakage is far from being elucidated. Among the factors implicated in chromosome breakage is the abnormal supply of energy for synthetic activities. Recently, it has been reported that phytohemagglutinin (PHA)-stimulated lymphocytes depend on glycolysis as the source of energy. In the presence of 2-deoxy-D-glucose, the syntheses of DNA, RNA and protein are inhibited. Since leukocytes depend on glycolysis for energy supply during "blast" transformation, it may be that there is an impairment of glycolysis in the leukocytes from CL $\pm$ CP patients, and that the higher frequency of chromosome breaks in their leukocyte cultures reflects this impairment in the glycolytic ability. In the absence of direct study of leukocyte glycolysis in these patients, the above remains strictly a speculation.

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References

1. AKSU, O., B. MACKLER, T. SHEPARD, and R. J. LEMIRE, Studies of the development of congenital anomalies in embryos of riboflavin-deficient, galactoflavin fed rats. II. Role of the terminal electron transport systems. *Teratology*, 1, 93-102, 1968.
2. CANICK, M. L., A review of embryology, pathologic anatomy, and etiology. *Plastic reconstr. Surg.*, 14, 30-45, 1964.
3. FOGH-ANDERSON, P., *Inheritance of Harelip and Cleft Palate*. Copenhagen: Arnold Busck, 1942.
4. FRASER, F. C., Genetics and congenital malformations. In *Progress in Medical Genetics* (A. G. Steinberg, ed.), pp. 38-80. New York: Grune & Stratton, Inc., 1961.
5. GROPP, A., A. JUSSEN, and F. ODUNJO, Near-triploid chromosome constitution in epithelial-cell cultures of palatal mucosa from a case of cleft palate. *Lancet*, 1, 1167, 1964.
6. JACKSON, J. F., Chromosomes in cleft-palate tissues. *Lancet*, 1, 1156, 1966.
7. LI, J. C. R., *Introduction to Statistical Inference*, p. 408. Ann Arbor: Edwards Bros., Inc., 1957.
8. MAKINO, S., Chromosomal studies in normal human subjects and in 300 cases of congenital disorders. II. *Cytologia*, 29, 125-150, 1964.
9. MOORHEAD, P. S., and P. C. NORWELL, *Chromosome Cytology: Methods in Medical Research*, 10, 310-322. Chicago: Yearbook Medical Publisher, 1964.
10. MOSES, L., Non-parametric methods. In *Statistical Inference* (H. M. Walker, and J. Lev, eds.), chap. 18. New York: Holt, 1953.

11. MURPHY, J. W., and L. E. REISMAN, Chromosomes in cleft-palate tissues. *Lancet*, *2*, 228-229, 1966.
12. NISWANDER, J. D., and M. S. ADAMS, Major malformations in relatives of oral cleft patients. *Acta Genet. (Basal)*, *18*, 229-240, 1968.
13. POLGAR, P. R., J. M. FOSTER, and S. R. COPPERBAND, Glycolysis as an energy source for stimulation of lymphocytes by phytohemagglutinin. *Exp. cell Res.*, *49*, 231-237, 1968.
14. SOUKUP, S., and J. WARKANY, Chromosomes in cleft-palate tissues. *Lancet*, *1*, 492-493, 1966.
15. SPRIESTERSBACH, D. C., BETTY R. SPRIESTERSBACH, and K. L. MOLL, Incidence of clefts of the lip and palate in families with children with clefts and families with children without clefts. *Plastic reconstr. Surg.*, *29*, 392-401, 1962.
16. SUBRT, I., J. ČERVENKA, and M. KRECEK, Cytogenetic study of cleft lip and palate. *Cleft Palate J.*, *3*, 362-367, 1966.
17. SWIFT, M. R., and K. HIRSCHHORN, Fanconi's anemia: inherited susceptibility to chromosome breakage in various tissues. *Annals int. Med.*, *65*, 496-503, 1966.
18. WARKANY, J., E. PASSARGE, and L. E. SMITH, Congenital malformations in autosomal trisomy syndromes. *Amer. J. dis. Child.*, *112*, 502-517, 1966.
19. WOOLF, C. M., R. M. WOOLF, and T. R. BROADBENT, A genetic study of cleft lip and palate in Utah. *Amer. J. hum. Genet.*, *15*, 209-215, 1963.