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Electron Optic Microanalysis of Two Gene Products in Enamel of Females Heterozygous for X-Linked Hypomaturation Amelogenesis Imperfecta

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The X-linked hypomaturation type of amelogenesis imperfecta was first described by Witkop [1–3] in two kindreds during a survey of 96,471 school children for defects of enamel and dentin. Witkop [4] added another kindred to the former and demonstrated the partial expression of the trait in presumed heterozygous females which was compatable with the Lyon [5] hypothesis. Presumed hemizygous affected males had an enamel of normal thickness but which was slightly softer than normal enamel with a mottled, opaque, ground-glass appearance (fig. 1*a*). The color varied from an opaque milky white in the primary dentition to a mottled yellow brown color in the permanent dentition. All the teeth in both primary and secondary dentitions were reported to be affected.

In females heterozygous for the gene, it was noted that varying degrees of vertically arranged bands of mottled enamel alternated with bands of normal appearing enamel in a random pattern (figs. 1*b*, 2). Both primary and secondary teeth in females exhibited this vertical banding. The defect was less severe in females than in affected males although individual teeth in females may have nearly all hypomature or all normal enamel. Witkop [4] attributed this pattern in females heterozygous for the gene to the cloning of ameloblasts carrying alternatively active X chromosomes as advanced by the hypothesis of Lyon [5]. If this hypothesis is applicable to this type of enamel defect, then two distinct gene products should be found in enamel of heterozygous females arranged in definite vertical clones.

The purpose of the present study was to investigate the ultrastructure and the chemical composition of enamel in an affected female and male. Such data, when

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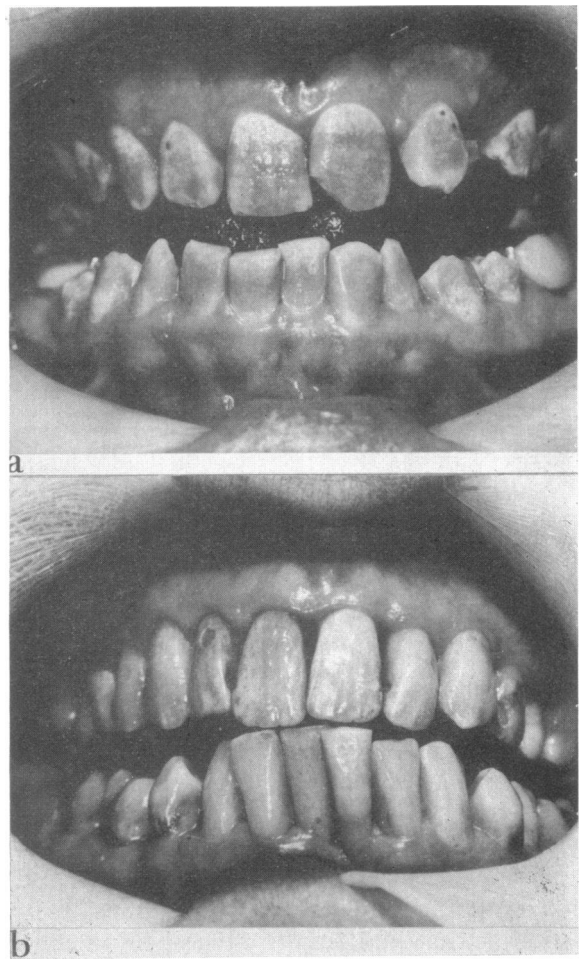

FIG. 1.—Fig. 1*a*, Dentition of the affected male. The teeth are mottled and yellow white colored. Enamel is of normal thickness but is softer than normal and can be penetrated by the point of a dental explorer. Fig. 1*b*, Dentition of the affected female with varying bands of affected and unaffected enamel, viewed with difficulty without transillumination.

compared with that from normal controls, could provide further evidence for the presence of two distinct gene products in the enamel of heterozygous females. In addition, it could provide information regarding the regulation of structure and chemical composition of enamel in general.

MATERIALS AND METHODS

A central incisor from an affected heterozygous female and a bicuspid from her affected son [4] were preserved in 10% neutral buffered formalin after extraction. In addition, clinically normal teeth extracted from healthy naval personnel were preserved and prepared in a similar way and used as controls.

Cross-section, fractured specimens were prepared with a mallet and chisel (fig. 3,

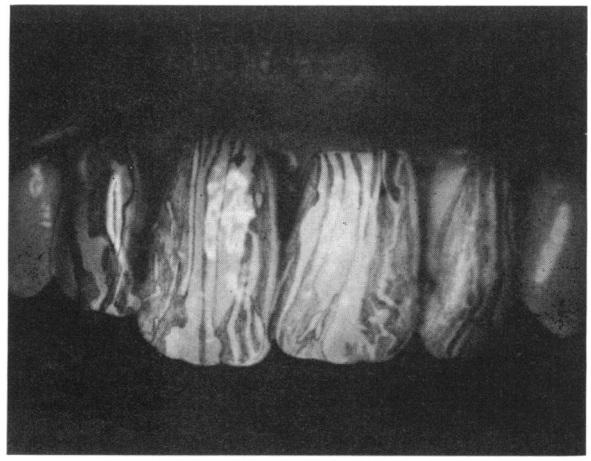

FIG. 2.—The contrast of opaque white and translucent white bands of enamel, being difficult to demonstrate, are depicted by a camera-lucide projection of the teeth of the affected female (fig. 1*b*) with a pencil overlay tracing of the defective enamel bands.

A). Half of the split specimen was polished with aluminum oxide and lapped with silk to a maximal relief of 1 μ. The other half was not polished. Half of the polished and unpolished specimens were placed in 0.12 M tetrasodium-ethylenediaminetetraacetate (EDTA), *p*H 7.8, for 1 hr. The remaining halves of the polished and unpolished specimens remained as controls. In addition, prism cross sections were prepared (fig. 3, *B*) by grinding with diamonds and were polished in a similar manner to the tooth cross sections. The EDTA etching enabled the investigators to observe greater detail on polished and unpolished surfaces with the scanning electron microscope (SEM) [6–9].

The specimens were glued on aluminum stubs and coated with approximately 200 Å of an electron-conductive mixture of carbon-gold palladium. They were examined with a Cambridge Steroscan microscope in the secondary electron mode at 10 kv. The results were recorded on Polaroid film (type 55 P/N) from a cathode-ray tube at 25 lines per second.

Similar cross-section specimens were cut with a diamond saw and mounted in E-Mount (Buehler Products, Evanston, Ill.) thermal setting resin for microprobe analysis

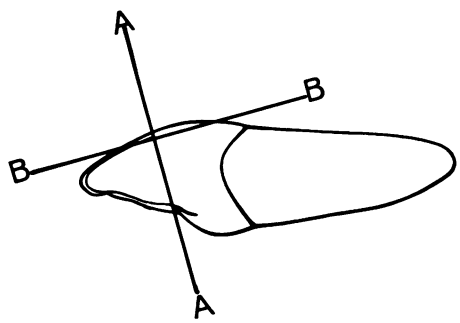

FIG. 3.—The planes of section are represented: (*A*), cross-sectional plane through the tooth; (*B*), approximate cross-sectional plane through enamel prisms.

of calcium and phosphorous. The specimens were polished such that a final relief of less than 1 μ was left on the surface [10]. They were coated with approximately 200 Å of an electron-conductive mixture of carbon-aluminum in a vacuum evaporator.

The teeth were then analyzed with a Cambridge Microscan V at 15 kv and a sample current of 10 na. The electron beam was approximately 1 μ in diameter when focused on the sample. The sample was scanned in 2-μ steps, and each step was counted for 40 sec. The samples were analyzed by focusing the beam 60 μ from the dentin-enamel junction and scanned horizontally across the enamel prisms.

RESULTS

Ground sections of teeth from the affected male revealed that a highly organized prism pattern was lacking and that the enamel was fibrillar in character. Decalcified sections revealed that an eosinophilic material was present on Masson's trichrome stain. As this material was traced peripherally, it enlarged and crossed rods forming an exclamation mark type of structure. The SEM scans of the sample of enamel from the male proband (fig. 4*a*) showed severe etching after 15 min of treatment with EDTA. Enamel prisms were difficult to establish as such and had a fibrous character. In addition, numerous spaces were located in the fibrous enamel. These spaces correlated with the areas occupied by the eosinophilic material seen on the Masson's trichrome. The spaces in the enamel varied in their distribution from small linear defects (fig. 4*a*, *A*) in what might be interprismatic regions, to areas extending into rods (fig. 4*a*, *B*) and large irregular spaces which extended across enamel rods (fig. 4*a*, *C*).

The SEM micrographs of the etched samples of enamel from the heterozygous female (fig. 4*b*) revealed two zones of enamel. These two zones correlated with the alternating vertical banding of the mottled and normal zones in the gross descriptions of the enamel defect (fig. 2). One of the zones was rather smooth and was relatively unetched with EDTA (fig. 4*b*, *A*), while the second zone of enamel was quite porous (fig. 4*b*, *B*), similar to the type of enamel seen in her son's teeth. The SEM scans of enamel in the heterozygous female prepared by grinding across the enamel prisms (fig. 3, *B*) revealed a highly porous zone of enamel (fig. 5, *A*) adjacent to a relatively unaffected zone (fig. 5, *B*). Once again, the distribution of these defects correlated with the vertical banding observed after overlay tracing of transilluminated enamel (fig. 2).

Comparisons of the calcium and phosphorous content by weight percent of the enamel from the heterozygous female, her male proband, and a group of healthy naval recruits, as analyzed by the electron microprobe, are given in table 1 and figure 6.

DISCUSSION

In 1961, Lyon [5] advanced the hypothesis that, in normal females, only one X chromosome per somatic cell is genetically active during interphase. The other X chromosome, represented by the Barr body (sex-chromatin body), retains its heterochromatic properties and hence is probably inactive. The hypothesis posits that, early in embryogenesis, each somatic cell of the female reaches a "time of

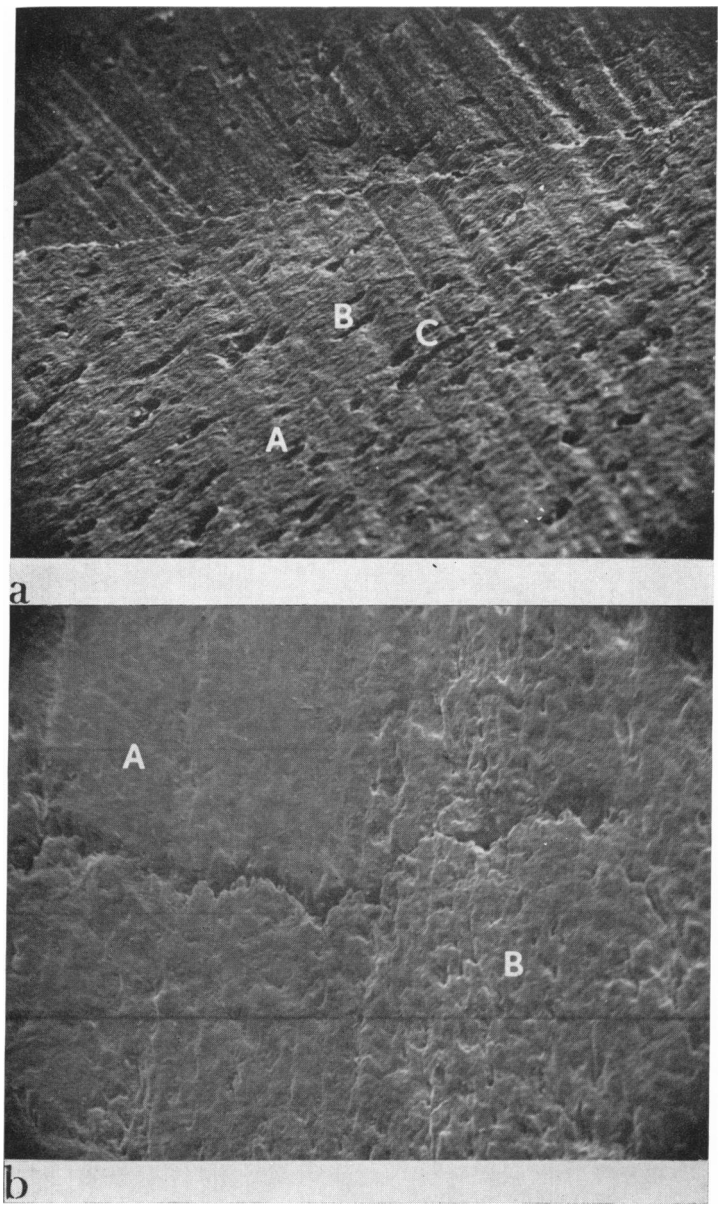

FIG. 4.—Fig. 4*a*, SEM micrograph from an affected male depicting a fibrous enamel with spaces. The spaces vary in distribution from small linear defects (*A*) in interprismatic regions, to areas extending into rods (*B*), and large irregular spaces which extend across enamel rods (*C*). Fig. 4*b*, SEM micrograph from the affected female showing two zones of enamel: (*A*) rather smooth unetched normal zone; (*B*) porous zone of enamel comparable to the affected enamel in the male. ×1,100; tilt angle, 45°.

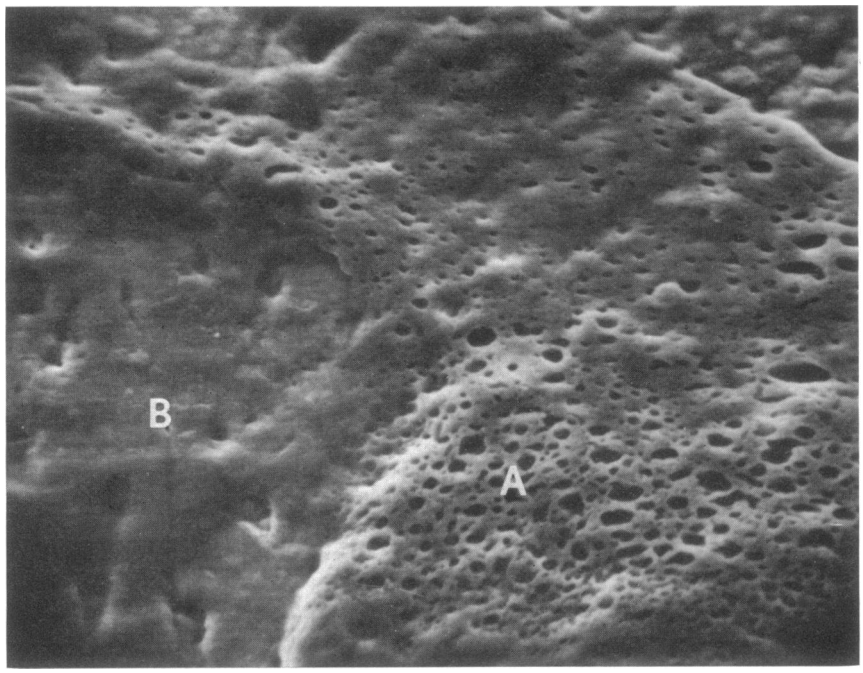

FIG. 5.—SEM micrograph from the affected female after the *B* type of sample preparation (fig. 3): (*A*), porous zone of dental enamel comparable to affected enamel in males; (*B*) unaffected enamel. The distribution of the defects correlates with that seen in the overlay tracing of transilluminated teeth (fig. 2). ×1,500; tilt angle, 45°.

decision" regarding whether a paternal X (X^p) or a maternal X (X^m) shall be the active chromosome in that particular cell. The hypothesis suggests that either the X^p or the X^m chromosome is selected at random. Once the decision is made, all the descendants of this embryonic cell retain the activity of the selected chromosome. Thus, females are a mosaic of cell clones with regard to activity of the X chromosomes in somatic cells [4].

TABLE 1

ELECTRON-MICROPROBE DATA FOR CALCIUM AND PHOSPHORUS

	WEIGHT PERCENT		
	Calcium	Phosphorous	Ca/P
Affected male	29.74 ± .35	14.30 ± .10	2.08 ± .04
Control (naval recruits)	36.16 ± .55	17.33 ± .26	2.08 ± .01
Heterozygous female (normal area)	35.94 ± .35	16.08 ± .20	2.15 ± .05
Heterozygous female (mottled area)	32.96 ± .58	15.24 ± .23	2.09 ± .03

NOTE.—The ± values indicate standard deviation from the mean.

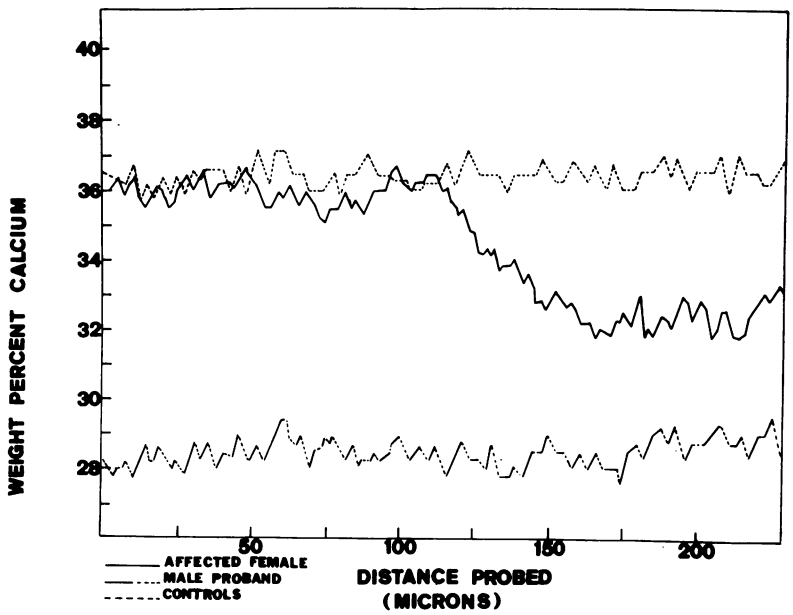

FIG. 6.—Levels of calcium in a comparable 225 μ of normal enamel, an affected male, and across two zones of differing enamel in an affected female. All scans were performed on polished cross sections of enamel scanned in 2-μ steps, and each step was counted for 40 sec. The samples were analyzed by focusing the beam 60 μ from the dentin-enamel junction and scanned horizontally across the enamel prisms.

The patterns of alternating vertical banding of normal and hypomature enamel, as demonstrated by heterozygous females, would be expected if the Lyon hypothesis is correct. Although the number of ectodermal cells at the time of decision destined to become ameloblasts is unknown, they probably represent only a few cells. Up to the time of morphogenesis, beginning with the bud stage, differential growth and migration would be expected to result in varying proportions of X^m- and X^p-containing cells in any one tooth. However, the total ameloblast population from the whole dentition would more closely reflect the proportion of X^p and X^m selected at the time of decision.

Once the outline form of the tooth begins, clone lines of inner-enamel cells, which eventually develop into ameloblasts, become fixed. As daughter cells of any one inner-enamel epithelial cell are added at the proliferating end of the dental organ, this clone line will have a vertical distribution in the completed crown. In essence, the type of alternating vertical banding of normal enamel and abnormal enamel demonstrated in such females could be interpreted as a record in the enamel of the particular X chromosomes governing different clone lines of ameloblasts.

These findings are supported by the SEM micrographs of enamel from a heterozygous female showing two types of enamel present: one type is well mineralized and relatively unetched by EDTA, and the other is a porous-appearing type of hypomature enamel. The electron microprobe also substantiates the SEM findings

that these two zones of enamel have different degrees of mineralization or maturation. However, it is difficult to explain why the affected zones in females do not approach the severity that their male probands manifest.

It appears that several explanations are possible. In addition to technical problems as a source of this difference, the possibility exists that the affected zones in females were not the pure clone lines of ameloblasts that was originally hypothesized, but at the micro level consist of some intermingling of two types of ameloblasts. Boyde's [11] hypothesis states that, in models of "type-three" prisms, two ameloblasts are related to each other in two planes, four ameloblasts per prism and four prisms per ameloblast. Thus, at least four ameloblasts are responsible for the formation and mineralization of one prism. If the vertical bands of affected enamel in females were not pure clones of "abnormal" ameloblasts, the development of an enamel prism may be altered but not to the same extent as the pure form of the disease. However, the enamel prism would still be abnormal. Closely allied to the former concept is the possibility that the normal primary gene product is diffusable and could be utilized by adjacent abnormal cells to partially correct the defect. Thus, in females having some normal cells, the normal product could be diffused via microcirculation to the abnormal cells incapable of primary synthesis of this constituent. The gradual rather than abrupt decrease in calcium content revealed by the probe in passing from one type of enamel to another, with the lowest calcium content being the farthest removed from the normal area, lends some support to either of the above possibilities.

It is also possible that the data could be interpreted as evidence for a codominant gene on the X chromosome with differing expression in females and in males, that is, the gene could be located in the non-Lyonizing part of the X chromosome. Thus, females would show the intermediate effects of both genes. However, Witkop [10] noted that some teeth of women showed nearly all hypomature enamel while others were nearly normal and that the bands varied in width and distribution, lending support to the idea that the gene is on the Lyonizing portion of the X chromosome. Further, since there is essentially no difference in the calcium content in the normal-appearing areas of the mother's enamel and that of normal controls, rather than an intermediate value expected under a codominant hypothesis, this fact can be interpreted as evidence against this explanation.

The enamel defect in the son's tooth is characterized by the presence of irregular-sized spaces involving the enamel sheaths, sheaths and rods, or groups of rods. The mother's enamel shows this type of defect in vertical clone-lined areas alternating with those areas which, by SEM and microprobe analysis, are identical to enamel from normal males. These findings are indicative of a mosaic represented by two distinct gene products in heterozygous females.

Therefore, it appears that there is at least one locus on the X chromosome that is involved in enamel formation. Two types of amelogenesis imperfecta have been reported to be X-linked traits. We do not know whether they are alleles or represent genes at two different loci. In addition to the hypomaturation type of amelogenesis imperfecta reported by Witkop [1–4], a hypoplastic form, first de-

scribed by Schulze [12], is known to be an X-linked dominant trait. Rushton [13] and Weyers [14] have described a similar alternating vertical banding of enamel of normal thickness with thin hypoplastic enamel in females heterozygous for the X-linked dominant hypoplastic amelogenesis imperfecta gene. Rushton [13] also illustrated similar teeth from a plaster model in the Odontological Museum of the Royal College of Surgeons in England. This was reported to be a model of an 8-year-old boy. Rushton discusses the possibility that the boy might have had Klinefelter's syndrome with an XXY karyotype. If so, the vertical pattern could occur in a male.

SUMMARY AND CONCLUSION

Enamel from a female heterozygous for X-linked hypomaturation amelogenesis imperfecta and her hemizygous son was investigated with both the scanning electron microscope (SEM) and the electron probe, and compared with enamel from normal males. The SEM micrographs revealed that enamel from the mother showed alternating vertically arranged bands of normal-appearing and porous hypomature enamel similar to her son. The microprobe analysis of these areas in enamel from the mother showed one zone which was less calcified than the other, but the affected zone was not as severely affected as the male proband. Possible explanations for this phenomenon, in addition to technical problems, are the possibility that migrations of normal and abnormal ameloblasts took place between zones to create a mixed population of cells within those zones, but with one type of cell predominating, or that, in the presence of some normal ameloblasts, the defect is partially corrected in the abnormal cells, possibly by a diffusable product. The evidence, however, substantiates the expectations of the Lyon hypothesis that two distinct gene products appear as different forms of enamel in a female heterozygous for the X-linked trait.

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