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Immunogenicity of Two Naturally Occurring Solid-Phase Enamel Proteins (39755)

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Introduction. Dental enamel is a highly mineralized tissue which forms the outer mantle of the functional portion of teeth. The tissue arises through the mineralization of a proteinaceous matrix, which in turn is secreted by an avascular epithelia (the "inner enamel epithelium"). Upon secretion by the inner enamel epithelium, the enamel proteins are precipitated upon the partially mineralized dentin matrix (1).

Since enamel proteins are secreted by an avascular epithelium, and since most newly secreted enamel proteins seem to be insoluble under physiological conditions (2), they may represent molecules which are "sequestered" from the immune system, and, as such, would be expected to be capable of eliciting immune responses if they were to be presented to a mature immune system. Solubilized enamel proteins have previously been shown to be immunogenic across species barriers (3, 4).

We are reporting the results of experiments designed to determine whether native, solid-phase embryonic enamel proteins obtained from New Zealand white rabbits could elicit an immune response in young adult female New Zealand white rabbits.

Materials and methods. Animals. New Zealand white rabbits were obtained from a commercial supplier (ABC Caviaries, Pomona, Calif.).

Antigens. Approximately 750 molar tooth extracellular matrices were prepared from embryonic rabbits of 26-days gestation by dissecting out the dental pulps and removing contaminating cell debris by sonication. At this stage of development, the acellular matrices contained solid-phase enamel proteins. Matrices were then pulverized in a liquid nitrogen mill and suspended in Freund's complete adjuvant at a concentration of 2 mg/ml.

Antisera. Young adult female New Zealand white rabbits were immunized with six consecutive weekly injections of 1 ml of the matrix-in-adjuvant suspension. Two weeks after the conclusion of the immunization program, the rabbits were bled and serum was prepared by ammonium sulfate precipitation (5). Control serum was prepared in an identical manner by injecting Freund's complete adjuvant without tooth matrix powder into another group of rabbits. Each group (experimental and control) consisted of three rabbits. The sera were not adsorbed at any time.

Immunofluorescence microscopy. Embryonic New Zealand white rabbit incisor teeth of 26-days gestation were prepared by dissecting out the dental pulps and removing cell debris as described. Frozen sections of 7-μm thickness were then cut parallel to their long axes on a cryostat. The sections were placed onto clean gelatin-coated microscope slides and thawed without fixation. Either experimental or control serum (both at concentrations of 0.67 mg of protein/ml) was then layered over the sections for 10 min at 37°. An additional control experiment was performed in which saline was substituted for the primary serum. The sera were washed off and the sections were treated with a fluorescein-conjugated antiserum to rabbit 7S γ-globulins, also for 10 min at 37°. Approximately 15 teeth in each of the three groups were treated in this manner. After washing and mounting coverslips, the slides were observed and photographed.

Immunoelectrophoresis. Embryonic New Zealand white rabbit incisor teeth of 26-days gestation were prepared as previously described. In a typical experiment, 160 acel-

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lular matrices were decalcified with a 5% trichloroacetic acid (TCA) solution for 30 min at 4°. The TCA was removed by washing with ice-cold methanol, and the tooth matrices were air-dried. Proteins were extracted from the matrix material by treatment with 6 *M* deionized urea in a 0.1 *M* Tris–borate buffer, pH 8.6, for 1 hr at 60° (2, 6). Approximately 160 μg of extracted protein, reduced with 400 m*M* mercaptoethanol, was electrophoresed on 7.5% polyacrylamide gels containing 6 *M* urea. Urea was washed out of the gels by placing them in several changes of a 0.1 *M* phosphate buffer, pH 7.0; only one rapidly migrating band (not associated with enamel protein) appeared to be lost as a result of this procedure. The gels were then treated with either experimental or control antiserum which had been labeled with ^{125}-I (7). Unbound antibody was washed out of the gels with many changes of the 0.1 *M* phosphate buffer, and the gels were sliced and counted in a liquid scintillation counter.

Results. Fluorescence micrographs showed binding of the antiserum over the areas of the tooth known to contain developing enamel (8) (Fig. 1). No binding of the antiserum to areas of the tooth composed of dentin was observed, even though dentin was present in the molar tooth material used for immunization and the serum was not adsorbed against dentin. No binding of the control serum or of the secondary (FITC-conjugated) serum was observed (9).

Further evidence for the production of antibodies against enamel proteins was afforded by immunoelectrophoresis. In these experiments, the acellular dentin and enamel matrices of embryonic teeth were chemically extracted with 6 *M* urea. After electrophoresis on 7.5% acrylamide gels, nine protein bands (plus some precipitated material at the tops of the gels) can be discerned. Three of these bands were previously identified as enamel specific (2, 6). The experimental, but not the control, serum was seen to bind to two of the three enamel protein bands resolved in this electrophoretic system (Fig. 2). Both experimental and control sera were noted to be bound to material at the top of the gels; this could be due either to nonspecific trapping of the sera in the band of precipitated protein found at the tops of the gels, or to the presence of naturally occurring autoantibodies against some material which migrated very slowly in this electrophoretic system (10).

Discussion. This report demonstrates that native, i.e., not chemically extracted or altered, insoluble, solid-phase enamel proteins present on embryonic tooth matrices were capable of eliciting antibody responses within the same strain of rabbit. The data were interpreted to suggest that an autoimmune reaction occurred in response to a "sequestered" antigen, even though the strain of rabbit used was not strictly inbred. An alternate explanation for the production of antibodies to the enamel proteins is that the responding animals were recognizing some heterogeneity of the enamel proteins found in different individuals of the strain. If the appearance of the antibody was due to this heterogeneity, one would expect such macromolecular heterogeneity to be reflected in the dentin proteins as well. However, no antibody production to dentin components was detected either by immunoelectrophoresis or immunofluorescence microscopy.

Another possibility is that tolerance to enamel proteins (but not to dentin proteins) was broken as a result of the immunization scheme, which employed complete Freund's adjuvant. Preliminary data generated in our laboratory seem to indicate that unprimed splenic lymphocytes from inbred stains of mice are capable of giving a lymphoproliferative response to syngeneic solid-phase enamel proteins *in vitro* (11). These experiments suggest that any tolerance to enamel proteins (if it exists) can be broken without the use of immunization programs dependent upon complete adjuvant.

A more plausible explanation for the antibody production is that enamel proteins, by virtue of their being secreted by an avascular epithelium and having low solubility properties, were not normally accessible to the immune system during the period of time in which tolerance to "self" antigens was achieved. The dentin-specific proteins, in contrast, were secreted by cells intimately associated with the vasculature (12) and hence were accessible to the developing immune system. Since the dentin proteins

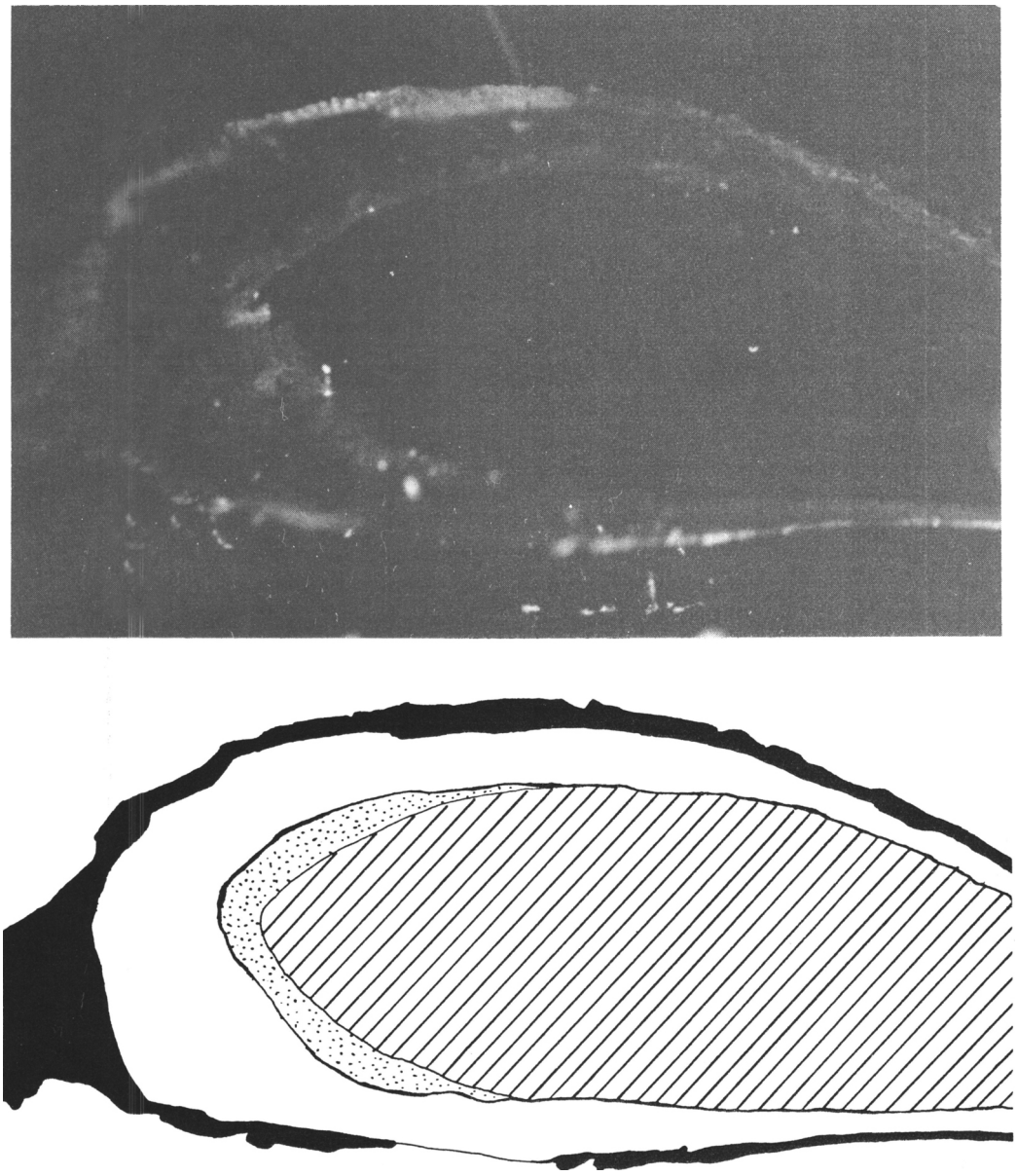

FIG. 1. Immunofluorescence photomicrograph (upper panel) showing binding of antiserum to embryonic solid-phase enamel proteins on the outer aspects of the section. The lower panel is a camera lucida tracing showing the various structures found in the fluorescence micrograph. The solid black areas represent the enamel matrix; the irregularity of this brittle matrix was due to fracturing during sectioning in the cryostat. The white areas represent dentin. Cross-hatched areas represent a void left when the dental pulp was removed, while the stippled areas represent some remnants of the pulp. Some binding of the secondary antibody (FITC-conjugated goat anti-rabbit serum) to endogenous rabbit γ-globulins present in the pulp can be seen in this area. Note that there is no binding of antiserum over areas of the tooth composed of dentin. All appropriate controls were negative.

were not sequestered, one would not expect antibody production in response to the dentin proteins. In fact, no antibodies to dentin proteins were found even though dentin proteins were present in the matrix used for the immunizations. Preliminary data generated in our laboratory also support our hypothesis by suggesting that enamel proteins

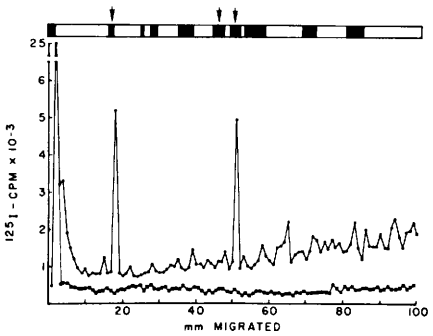

FIG. 2. Immunoelectrophoretic patterns compared with a diagram of a urea–polyacrylamide gel. The positions at which three enamel-specific protein bands are known to migrate are indicated by arrows. Both experimental (circles) and control (squares) sera bound to material at the tops of the gels (see text). The experimental, but not the control, serum was seen to bind to areas of the gel coincident with the positions at which two of the three enamel-specific proteins migrate. These radioactive peaks were noted to occur in single 1-mm slices on all four repititions of the experiment; in addition, fine precipitin bands were noted in these areas of the experimental gels when they were stained prior to slicing and counting. Specific activities of the sera used were: experimental, 4.3×10^4 cpm/mg of protein; control, 1.0×10^5 cpm/mg of protein.

from inbred strains of mice are capable of eliciting autoimmune responses both *in vitro* and *in vivo* (11).

Summary. An antiserum to native, solid-phase, insoluble enamel proteins from New Zealand white rabbits was raised in the same strain of animal. The ability of the antiserum to bind enamel proteins was demonstrated by fluorescence and radioimmunoelectrophoretic techniques. The data were interpreted to suggest.that enamel proteins are sequestered from immune surveillance during early development, resulting in subsequently induced autoantibody production.

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