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Identification of Immunoreactive Material in Mammoth Fossils

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Abstract

The fossil record represents a history of life on this planet. Attempts to obtain molecular information from this record by analysis of nucleic acids found within fossils of extreme age have been unsuccessful or called into question. However, previous studies have demonstrated the long-term persistence of peptides within fossils and have used antibodies to extant proteins to demonstrate antigenic material. In this study we address two questions: Do immunogenic/antigenic materials persist in fossils? and; Can fossil material be used to raise antibodies that will cross-react with extant proteins? We have used material extracted from a well-preserved 100,000-300,000-year-old mammoth skull to produce antisera. The specificity of the antisera was tested by ELISA, western blotting, and immunohistochemistry. It was demonstrated that antisera reacted specifically with the fossils and no the surrounding sediments. Reactivity of antisera with modern proteins and tissues was also demonstrated, as was the ability to detect evolutionary relationships via antibody-antigen interactions. Mass spectrometry demonstrated the response of amino acids and specific peptides within the fossil. Peptides were purified by anion-exchange chromatography and sequenced by tandem mass spectrometry. The collagen-derived peptides may have been the source of at least some of the immunologic reactivity, but the antisera identified molecules that were not observed by mass spectrometry, indicating that immunologic methods may have greater sensitivity. Although the presence of peptides and amino acids was demonstrated, the exact nature of the antigenic material was not fully clarified. This report demonstrated that antibodies may be used to obtain information from the fossil record.

Key words: fossil, mammoth, ancient proteins, molecular preservation, mass spectrometry

Introduction

The history of life on this planet is written both in living organisms and in the fossilized remains of extinct organisms. Although fossils provide excellent morphologic data, convergence of traits may obscure some evolutionary relationships. A better understanding of the evolutionary relationships among extant and extinct species can be attained by combining morphological and molecular analyses. Despite inventive fiction and public fascination, attempts to derive molecular information from nucleic acids within the fossil record have been largely unsuccessful and laden with artifact, stemming from contamination with microbial and fungal DNA from soil organisms (Sidow et al. 1991) and from human DNA (Hedges and Schweitzer 1995). Moreover, it is unclear whether nucleic acids can withstand the digenetic alterations associated with the process of fossilization (Lindahl 1993). Thus it is necessary to develop alternative approaches to explore the possibilities of recovering molecular information from fossils.

It is well established that amino acids and short peptides can survive within fossils for millions of years (My^3) (Weiner et al. 1976; Westbroek et al. 1979; Armstrong et al. 1983; Muyzer et al. 1992; Collins et al. 1991; Schweitzer et al. 1997a, b, 1999a, b). If present in at least femtomole quantities, these peptides may be suitable for analysis by tandem mass spectrometry (MS/MS) (Stankiewicz et al. 1996, 1997). However, this is not always the case, as the peptides may have been altered, cross-linked, or otherwise chemically modified in the process of fossilization, thus limiting the utility of mass spectrometry (MS) or other analytical methods (Mycke and Michaelis

1985; Rafalska et al. 1991; Poinar et al. 1998). An alternative approach applied in this study and others is to use antibodies made to proteins of extant animals to identify and study antigenic material in fossils (Schweitzer et al. 1999a, b; Rowley et al. 1986; Muyzer and Westbroek 1989; Lowenstein and Scheuenstuhl 1991; Nerlich et al. 1993; Franc et al. 1995; Borja et al. 1997). Presumably peptides found within the fossils are antigenic material, although this has not been proven.

In this paper we demonstrate that extracts of fossils themselves can be used to produce antibodies that are fossil specific. We have chosen to study a well-preserved mammoth skull [Rancholabrean, Middle or early Late Pleistocene (Hill and Schweitzer 1999)]. The target antigens were identified using extant proteins, and the distribution of antigen within the fossils was studied by immunologic staining. Western blot analysis suggested chemical modification of the fossil antigens in both cases. Evolutionary relationships were examined by testing the cross-reactivity of the anti-fossil sera with extracts of extant bone tissues. The specificity and sensitivity of immunoassay and microcapillary liquid chromatography tandem mass spectrometry (LC/MS/MS) were compared. MS/MS sequencing confirmed the identity of collagen-derived peptides, but antibodies to other antigens not identified by MS were also present. The nature of the antigenic material in fossils is discussed.

Materials and Methods

Fossils, Modern Tissues, Purified Proteins, and Enzymes

An adult specimen of *Mammuthus* cf. *M. columbi* (MOR 604) was recovered from Pleistocene terrace (fluvial) sequences (Doeden gravels) of eastern Montana. The quarry was located at a level that allows for comparison and correlation with nearby Tongue River sediments that have been dated by uranium series to a minimum age of 0.1 m.y. and a maximum age of 0.3 m.y. (Hill and Schweitzer 1999). Sediments recovered from within bony cavities of the skull provided negative controls. A second, younger (approx 11,000 years) mammoth specimen (MOR 501) was also studied.

Naturally weathered bone from the following species was also used to compare with mammoth bone: elephant rib, domestic cat, coyote jaw, deer metatarsal, long bone shaft from rabbit, trabecular (spongy) bone taken from horse femur, and femur cortical bone from a freshly killed cow. A snake vertebra and a bird limb bone were also extracted for analyses. Bovine collagen I, bovine osteocalcin, and human thyroglobin were purchased from Sigma Chemical Co. (St. Louis, MO), and horse hemoglobin from Biogenesis (Kingston, NH). Proteinase K and collagenase (clostridiopeptidase, No. C9891) were obtained from Sigma Chemical Co.

Extraction of Antigens and Immunizations

Samples of ancient or extant bone were first ground with a sterilized Dremel tool to remove all surface material that may have been exposed to contamination, either from sediment influences or from human handling. Tissues were ground to fine powder and washed with a sterile 0.5. *M* NaCl solution, then rinsed multiple times in a sterile d H_20. Samples were centrifuged to pellet the bone powder, and the wash solution was removed. An extraction buffer consisting of 1.2 g/ml guanidinium thiocyanate, 0.1 *M* Tris/HCl (pH 5.4), 2 *M* EDTA (pH 8.0), and 26 mg/ml Triton detergent was added to the washed bone powder and extracted in this buffer overnight at a 60°C with gentle agitation. Samples were centrifuged, and supernatants were collected and added to dialysis tubing (2000 molecular weight cutoff). The supernatants were dialyzed with several changes for 48 against either phosphate-buffered saline (PBS) or distilled water at 4°C with stirring. The dialysis was then lyophilized to completion under vacuum. As negative controls, sediments or extraction buffer alone was extracted in parallel with the bone extracts. For one experiment, a small portion of the extracted mammoth material was incubated in a 0.1% solution of triflouroacetic acid (TFA) overnight, and then tested by immunoblot with the anti-mammoth antibodies.

Lyophilized fossil extracts were solubilized in sample buffer at a 1:1 dilution and electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Silver staining revealed smears of material consistent with degraded organic material (not shown). These extracts were then used to immunize rabbits. To generate mammoth antibodies, primary injections consisting of 7 mg lyophilized bone extract in Freund's complete adjuvant were given, followed by two boosters in Freund's incomplete adjuvant 2 weeks apart.

Chemical and Physical Characterization of Fossils

Small fragments of the mammoth skull bone fragments, as well as fragments of cortical tissues from a piece of elephant rib, were subjected to scanning electron microscopy (SEM; JEOL 6000 scanning EM coupled to a Noran Voyager energy-dispersive X-ray system). Whole samples were coated with 10-20 nm of carbon and visualized at 15 kV at various magnifications. Elemental analyses of mammoth and elephant rib were performed using energy-dispersive X-ray probe analysis on intact bone fragments.

Amino acid analyses were performed on the mammoth bone extracts. Lyophilized mammoth bone and sediment extracts were hydrolyzed in 6 *N* HCl at 110°C for 18 h and subjected to amino acid analysis on an Applied Biosystems 420A analyzer by phenylthiocarbamyl derivatization. Because the bone extract showed a strong hydroxyproline signal that was absent in the sediment extract, the results were submitted to a compositional database search using AaCompIdent (http://expasy.proteome.org.au/tools/aacomp).

Ion Trap MS/MS Sequencing

After reduction and S-carboxyamidomethylation, mammoth bone and sediment extracts were digested separately with trypsin (Promega, Madison, WI) and a 10% aliquot of each sample was cleared of nonionic detergent (Triton-X) using a weak anion-exchange resin immobilized inside a 10 µl pipette tip ZipTip$_{AX}$; Millipore, Bedford, Ma). The ZipTip$_{AX}$ was equilibrated three times with 10 µl of 50 mM diammonium citrate (Fluka, Milwaukee, WI). The sample was bound to the column by mixing 3 µl of sample with 7 µl of 50 m*M* diammonium citrate and cycling 10 times with the equilibrated ZipTip$_{AX}$. Bound sample was washed with high performance LC (HPLC)-grade water (J.T. Baker, Phillipsburg, NJ) and 20% methanol (J.T. Baker). Peptides were eluted from the tip of 3 µl of 2% acetic acid, and 7 µl of 1% acetic acid was added to the elution to acidify the solution prior to microcapillary LC/MS/MS. Multiple peptide sequences were determined in a single run by reverse-phase chromatography coupled directly to a Finnigan LCQ quadrupole ion trap MS equipped with a custom nanoelectrospray source. The column was packed in-house with 10 cm of C_{18} support into a New Objective (Cambridge, MA) one piece 75-µm- I.D. column terminating in an 8.5-µm tip. During chromatography, the ion trap repetitively surveyed full-scan MS over the range of 300-1400 *m/z*, executing data-dependent zoom and MS/MS scans on the three most abundant ions for charge state determination and peptide sequence information. The resulting MS/MS spectra were correlated with known sequences using the algorithm SEQUEST (Eng et al. 1994) and confirmed with programs developed at the Harvard Microchemistry Facility (Chittum et al. 1998).

Immunological Methods

Enzyme-linked immunosorbent assay (ELISA) was performed by coating polystyrene microtiter wells (Immulon 2HB; Dynatech, Chantilly, VA) with extracts of mammoth or extant bone extracts or sediment at 10 µg/ml overnight at 4°C or for 4 h at room temperature. Plates were then blocked with PBS/1% bovine serum albumin or with PBS/1% dried milk/0.1% Tween-20 (blotto) for a minimum of 18 h. Test antisera were added to the wells and incubated overnight at 4°C. Sera were then removed, the plates were washed 6x with PBS/1% Tween-20 , and alkaline phosphatase-conjugated anti-rabbit Ig (Zymed, South San Francisco, CA) was added. Following incubation and washing steps, the colorimetric substrate *p*-nitrophenyl phosphate (Sigma) was added at 0.5 mg/ml. The absorbance at 405 mm was monitored at various time points thereafter. The data presented are the means of multiple determinations, with error bars indicating the standard error of the mean (SE). When no error bars are present, the SE was smaller than the symbol used in the graphic.

Enzymatic digestion prior to ELISA was also performed as follows. Lyophilized elephant and mammoth extract were solubilized to 1 mg/ml in a solution of 25 m*M* Tris/5 m*M* $CaCl_2$. From this solution, 150 µl was added to proteinase K at varying dilutions (1.0, 0.1, and 0.10 µg/ml), and to collagenase at 10 mg/ml. The samples were digested for 3 h at 37°C or overnight at room temperature. Following, digestion, antigens were either diluted to 10 µg/ml for ELISA or electrophoreses onto SDS-PAGE and transferred to nitrocellulose membranes for probing with polyclonal anti-mammoth sera.

In addition, extracted and lyophilized material was digested with other enzymes as follows, to test the probability that these components contributed to the immunogenicity of the extract. Lyophilized mammoth and elephant extracts were solubilized in enzyme buffer, and either RNase A (Sigma R 5503) or DNase I (Sigma D 7291) was added at two concentrations. To test if the majority of the immunogenic material was carbohydrate, both bone

extracts were incubated with either amyloglucosidase (Calbiochem 172425) or β-glucosidase (TCI catalog No. G 035) according to the manufacturer's recommendations.

Immunoblots were performed by taking the fossil, bone, sediment, or purified proteins, boiling them in sample buffer [0.5 *M* Tris (pH 6.8), 10% SDS, glycerol, bromphenol blue, 0.28 μg/ml β-mercaptoethanol (BME)] and running them on 12% SDS-PAGE gels. The samples were then electrophoretically transferred to nitrocellulose membranes. The membranes were incubated with blotto and then with the primary antiserum. Following washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit Ig (Zymed) and then with the chemiluminescent substrate (Pierce Supersignal West Pico Chemiluminescent Substrate). Images were developed on X-ray film (Biomax ML; Kodak, Rochester NY).

To perform *in situ* immunohistochemistry, small fragments of mammoth or elephant bone were subjected to partial decalcification in 0.5 *M* EDTA (pH 7), and then dried at increasing concentrations of ethanol (50, 70, 100%). After air-drying for 2 days, these fragments were embedded in LR White, a water-soluble medium designed for immunochemistry. Sections of 0.5 μm thickness were taken with a glass or diamond knife, applied to gelatin-coated slides, and allowed to dry. Sections were etched three times for 10 minutes each in 1 mg/ml sodium borohydride, then blocked in 4% normal goat serum diluted in sterile PBS. Either preimmune or test sera were allowed to incubate with the sections overnight. Sections were then washed, and secondary antiserum (biotinylated goat anti-rabbit IgG; Vector Labs Inc., Burlingame, CA) was applied. Finally, sections were incubated with fluorescent label (avidin-FITC; Vector Labs Inc.), washed as before, and visualized using a BioRad DVC 250 confocal microscope equipped with an argon-krypton laser and a Photonics cooled color integrating CCD camera. All data were integrated, and images were captured using NIH Image software.

Results

Physical and Chemical Characterization of Mammoth Fossils

We have hypothesized that the degree of chemical, and therefore immunological, preservation of fossils will correlate with the physical preservation. Scanning electron micrographs were performed comparing the morphology of the mammoth fossil and an elephant rib bone (Fig. 1). At this magnification, very little alternation of the mammoth bone is seen compared with the 20-year-postmortem elephant bone and no recrystallization or alternation of bone matrix is evident.

Figure 2 shows a comparison of elemental profiles obtained from extant elephant and mammoth bone. Iron and calcium are slightly increased in the mammoth bone, relative to the elephant, while carbon and sodium are slightly reduced. In the mammoth, there are slight but measurable peaks for fluorine and silicon, while none is detected in the elephant. These elemental profiles indicate that there has been some chemical degradation and alteration in the mammoth that has not occurred in the elephant. However, the overall profile is similar between the two specimens. Carbon and oxygen have been included in these data; however, estimates of element percent may not be highly accurate for these elements because (1) they are ubiquitous, and the energy-dispersive X-ray analysis is not capable of distinguishing between endogenous and exogenous carbon or oxygen, and (2) quantification of the lighter elements is less accurate than for heavier ones.

Table 1 lists the amino acids identified by reserve phase HPLC analysis of the mammoth bone extracts. They are compared with amino acids from the sediments taken from within the skull cavities and extracted in parallel with bone. The amino acid percentages differ between the two samples, and the amino acids in the bone extracts are greater by an order of magnitude than those present in sediments. This, in addition to the relative content of amino acids consistent with collagen (i.e., 34% gly, 7% hyp), and the identification of a strong hydroxyproline peak in HPLC separations (data not shown) indicate that they are endogenous to the bone and do not arise from exogenous contamination.

Table 2 lists the peptide sequences obtained by tandem mass spectrometry and SEQUEST analysis of the trypsinized bone extracts. SEQUEST consistently correlates (Xcorr > 2.7, Sp > 500) the tandem mass spectra (MS/MS) with peptide sequences derived from collagen I α-chain. MS/MS spectra from identically analyzed sediment extracts revealed no significant peptide sequence. While the databases consistently identify these peptides as derived from collagen I, there are no sequences in the databases from extant animals phylogentically close to mammoth, and

therefore close matches to a specific taxon are not identified. We believe that these sequences represent the oldest peptide sequences yet obtained from fossil material.

Production of Anti-Mammoth Antisera

Material extracted from the mammoth fossil was used to immunize rabbits. Although the amount of immunogen may seem excessive (7 mg per immunization), it is likely that only a small proportion of this material is the actual antigen. The resulting sera were tested by ELISA for binding to mammoth or elephant extracts. The antiserum shows the greatest reactivity with the mammoth extract used as immunogen, then with extract from a second, younger mammoth (not shown), and next with elephant. Preimmune serum does not bind at all (Fig. 3A). The binding is dependent upon the concentration of antigen used to coat the well (Fig. 3B). Immunoblots were performed to probe the specificity of the antibody binding (Fig 4). The preimmune serum binds neither to the fossil extract nor to material extracted from the sediments found adjacent to the fossil. Immune serum does not bind to the sediment extract but produces a smear in the lane containing the fossil bone extracts. This reactivity is found in the molecular range of approximately 6.5-120 kD and suggests that the immunoreactive material either has been chemically modified and cross-linked, possible by humic substances within the soils, or is a diffuse mixture of heterogeneous molecules. To determine which is likely, the mammoth extract was first incubated with 0.1% TFA overnight at room temperature and then run on the gel. The resulting resolution of the pattern into identifiable bands suggests that the original material was chemically modified.

Characterization of Anti-Mammoth Antisera

The specificity of the binding of anti-mammoth serum was tested by competitive inhibition ELISA. Antiserum was premixed with varying concentrations of mammoth or elephant extract or as a control thyroglobin (a protein that would not be found at significant concentrations in any bone tissues). The data (Fig. 5) demonstrate that the antiserum has a significantly higher avidity for mammoth than elephant extracts. This was consistent across repeated experiments, including ELISA and immunoblot studies (data not shown).

To determine whether the material identified by the antibodies is proteinaceous in nature, the mammoth and elephant extracts were incubated with either proteinase K or collagenase and then used as antigens to coat ELISA wells (Fig. 6). While the antigenic material in the elephant was largely digested by the proteolytic enzymes, the mammoth material was unaffected. The failure of the proteases to destroy the mammoth antigens may indicate either that the antigens are not derived from protein, that they have been so extensively modified that they cannot be digested, or alternatively, that there is an inhibitor of the proteases in the fossil extract. However, the digestion of the cross-reactive material in the elephant extracts strongly suggest that the antigenic structures were originally proteins, as a similar reduction in signal was not seen when elephant was digested with other enzymes (data not shown). An immunoblot performed on collagenase digested mammoth, elephant, and cow extracts yielded results similar to those observed in the ELISA (not shown). When the mammoth and elephant material was digested with enzymes specific for DNA, RNA, or carbohydrates, no comparative decrease in signal was seen.

To characterize further the antigen(s) identified by the anti-mammoth antiserum, immunoblots were performed using purified proteins (Fig 7). Immunoreactivity is clearly seen with collagen, but also with osteocalcin and hemoglobin. This reactivity is not seen in preimmune serum.

To determine whether evolutionary information may be obtained using antisera to fossils, extracts were made from weathered bones of different taxa. These extracts were used as antigens in ELISA (Fig. 8). Mammoth antibodies reacted with decreasing strength when reacted with elephant extract then with horse, followed by other mammals, and the least with birds and snakes. Immunoblots yield similar results (not shown). Immunologic cross-reactions between extant and extinct species can indicate evolutionary relationships.

Figure 9 shows the immunohistochemical localization of antibody signal to sections of decalcified mammoth and elephant bone. The preimmune sera show no reactivity with demineralized bone sections, while strong and localized reactivity of the immune serum is seen with both mammoth and elephant bone. Because the major bone protein is collagen, it is very likely that the majority of staining reflects antibody binding to collagen structures. The elephant bone is more reactive than the mammoth bone to the sera, suggesting better preservation of antigenic material in the immunological reactivity of specific structures within the bone rather than due to an infiltration of contaminants from the surrounding sediments.

Discussion

In this paper we have shown that antigens found within fossils can be used to elicit antibodies that react specifically with fossils and cross-react with antigens found in similar tissues of closely related species. We have used tandem MS/MS to identify peptides in the mammoth that conform to the observed extant immunologic reactivity. However, the antibodies react with a broader range of molecules than is identified by MS. Although we have identified peptides within the mammoth, some of the antibodies clearly react with protein antigens; it is not proven that the antigens within these fossil extracts are peptides. This is the first demonstration that preserved antigens in fossils of this antiquity can be used to elicit antibodies and that these antibodies can be used to derive phylogenetic information. We also believe that the peptides we have sequenced represent the oldest protein sequence derived from fossil material.

The possibility of bimolecular preservation in ancient tissues received serious consideration when, in 1974, DeJong et al. (1974) demonstrated the retention of the antigenic components of proteins by immunoprecipitation within the shells of mollusks that could be dated to approximately 70 MY NP (before present). Others (Weiner et al. 1976; Westbroek et al. 1979) subsequently showed that endogenous biomolecules corresponding to structural proteins remained within the matrices of invertebrate shells. This early work was taken further when the presence of proteins and/or amino acid protein constituents was confirmed through the use of sensitive amino acid analyses (Armstrong et al. 1983; Gurley et al. 1991).

Lowenstein (1981, 1985, 1988) demonstrated antigenic reactivity in fossil bones using solid phase radioimmunoassays, identifying protein acetous compounds in a variety of bone material, including human, which dated to 2 MY BP, paving the way for utilizing such immunological methods with fossil bone to elucidate phylogenetic relationships (Lowenstein 1985, 1988). Immunological methods have since confirmed the existence of antigenic material in fossils of varying ages and from various source taxa (e.g., Muyzer et al. 1992; Collins et al. 1991; Schweitzer et al. 1997a, b, 1999a, b; Tuross 1989; Baird and Rowley 1990).

Collagen I has become one of the most studied proteins present in fossil tissues (DeNiro and Weiner 1988; Tuross 1989; Baird and Rowley 1990; Schaedler et al. 1991; Bocherens et al. 1997; Semal and Orban 1995), because bones and teeth are most likely to be preserved in the fossil record, and collagen is the most prevalent protein in these elements (Van der Rest 1991). Collagen in bone is resistant to degradation in part because of its molecular structure. Its three intertwined helices contain polar and non-polar residues, and its side-chain characteristics and post-translational modifications give collagen a strong affinity for the bone mineral, hydroxyapatite, which in turn conveys a high potential for preservation (Van der Rest 1991). Based upon amino acid profiles or immunological results, collagen fragments or degradation products have been identified in several ancient bone samples (Ho 1966; Wyckoff and Davidson 1976; Jope and Jope 1989; Baird and Rowley 1990; Tuross and Stathopoulos 1993; Tuross 1994), including dinosaurs (Schweitzer et al. 1997a, b).

It was soon recognized that in addition to collagen, proteins such as IgG, hemoglobin, and albumin (Lowenstein 1988; Cattaneo et al. 1990) could be identified in appropriate fossil specimens. In addition, the vertebrate-specific protein osteocalcin has been identified from bone and tooth samples (Urlich et al. 1987) including those of dinosaurs (Muyzer et al. 1992). The persistence of molecular fragments in fossil tissues has raised the hope that evolutionary traits, phylogenetic relationships, the timing of the origin of molecular evolutionary novelties, and other valuable information can someday be obtained directly from studies of fossil organisms, rather than relying on interpretations based upon assumptions derived from the study of their living descendants.

The most serious hindrance to identifying endogenous molecules within fossils is exogenous contamination. In this study, we address this issue in the following ways. First, we demonstrate that the antibodies raised against mammoth bone do not react with extracts of the adjacent sediments. Second, we demonstrate that antibody reactivity is greatest with robust bone-derived proteins, such as collagen and osteocalcin, and no antibody reactivity was demonstrated when the antibodies were tested against irrelevant (non-bone-derived) antigens. Third, antibody signal is localized to sections of decalcified specimens, and the patterns are consistent with antibody binding to extant samples.

Still unresolved is the question of the nature of the antigenic/immunogenic material. As mentioned above, the presence of peptides, as well as the mammoth antiserum reactivity with modern proteins, demonstrates that at least some of the material derived from fossils is proteinaceous. The lack of effectiveness of digestive enzymes on the fossil material relative to the extant samples suggests the possibility that some of the antigenic material is not protein

or so extensively modified as not to be digested by proteases. Such modifications may include intra- and intermolecular cross-linking, loss of side chains, polycondensations, methylation, and other modifications to the primary structure. Additionally, source proteins may be incorporated into diagenetic products of degradation, both of vertebrate proteins and of other organic sources, which inhibit enzyme reactivity (Handt et al. 1994). Chemical modification of the antigens in this study is supported by immunoblot analyses, which demonstrate a wide range of molecular mobilities rather than distinct molecular species. Finally, it is possible that some antigens are preserved through the process of molecular imprinting (Mosbach 1994) or atom-for-atom replacement during the fossilization process. This process would retain the three-dimensional shape of the original antigens, while retaining very little of the original organic material.

We also wish to note here the variation seen in reactivity of the antiserum to various extracts done over the course of the 2 years of this study. Microenvironments within fossil bone can vary greatly, and we believe that this is reflected in the variation of some of our results.

In summary, we have shown that antibodies to endogenous antigens of fossils can be made. Although the original molecules from which the antigens derive appear to be proteins, and peptides can be identified within the fossils, the acts chemical nature of the antigens was not elucidated. Future studies will use immobilized and monoclonal antibodies to obtain a better definition of the antigenic structures. Additionally, these studies suggest that antibody-antigen interactions can be applied to a wide range of fossil material, not only to demonstrate the persistence of endogenous molecules in fossil tissues, but also to purify and isolate specific components from a heterogeneous mix of organic compounds for further and more specific characterization of these compounds.