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IN VIVO CALCIFICATION INDUCED BY A PROTEOLIPID COMPLEX (LYSOZYM--ETC(U)
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sites greatly exceeded the rate at the control sites ($p<0.0005$). By 21 days the experimental areas still showed an accelerated healing response compared with the controls ($p<0.005$). By 28 days the experimental and the control wounds demonstrated almost complete osseous healing.

IN VIVO CALCIFICATION INDUCED BY A PROTEOLIPID COMPLEX (LYSOZYME-ACIDIC PHOSPHOLIPID)

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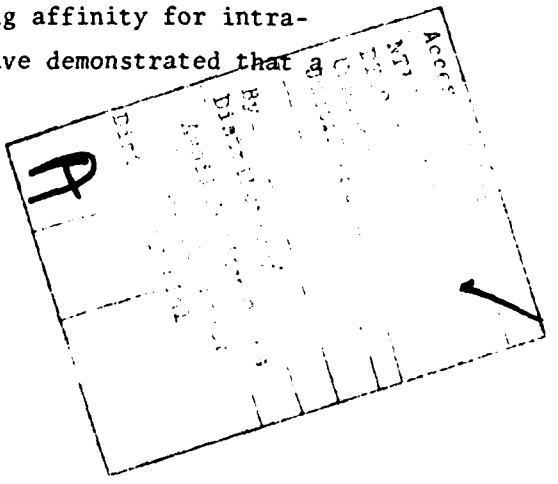
ABSTRACT

A synthetically prepared proteolipid complex was inserted into experimentally produced osseous wounds in the tibias of rats. Similar control wounds were made in the humeri and no proteolipid was inserted. At 7-day intervals for 28 days, rats were sacrificed and both the control and experimental sites were evaluated by gross inspection after the overlying soft tissues had been surgically removed. Specimens were then processed for histologic evaluation employing a Zeiss Videoplan Image Analysis System with Osteoplan™ for quantitative bone morphometrics. At the 7 and 14 day levels, the rate of osseous wound healing at the experimental sites greatly exceeded the rate at the control sites ($p<0.0005$). By 21 days the experimental areas still showed an accelerated healing response compared with the controls ($p<0.005$). By 28 days the experimental and the control wounds demonstrated almost complete osseous healing.

INTRODUCTION

Considerable attention has been focused on the important role that certain acidic phospholipids play in the process of calcification.[1,2,3] It appears that the acidic phospholipids possess both a calcium binding capacity and a strong affinity for intramembranous proteins.[4,5] Several studies have demonstrated that a



proteolipid complex can initiate both bone and calculus matrix calcification *in vitro*.[6 7] To date, no investigation has been undertaken to determine if a proteolipid complex (lysozyme-acidic phospholipid) can induce osteogenesis *in vivo*.

METHODS AND MATERIALS

A commercially manufactured lysozyme (mucopeptide-N-acetyl-muramoylhydrolase, E.C. #3.2.1.17) was checked for purity using disc gel electrophoresis. The acidic phospholipid, diphospho-inositide (phosphatidyl inositol 4,5 diphosphate), was examined for purity by thin layer chromatography.

The lysozyme and diphosphoinositide were dissolved separately in 0.12 N aqueous sodium chloride at a concentration of 1 mg/ml. Each solution was filtered through sterile Swinnex-25 units with type HA filters. The lysozyme solution was titrated with the phospholipid solution until a chalky white precipitate was produced. The volume ratio of lysozyme to phospholipid was approximately 2:1. After standing for 30 minutes at 25°C, the lysozyme-phospholipid solution was spun at 12,000 g in a centrifuge for 5 minutes at 5°C. The precipitate that was recovered was resuspended and washed two times with deionized water. The proteolipid complex produced was blotted and placed between two pieces of sterile #50 Whatman filter paper and stored at -40°C.

Sodium pentobarbital, USP (pentobarbital sodium) at a dosage of 3 to 5 mg/100 mg of body weight was administered intraperitoneally to anesthetize 20 Walter Reed strain of rats. The experimental sites (tibias) receiving the proteolipid complex and the control sites (humeri) were clipped and scrubbed with povidone iodine, N.F. (Betadine®) for 5 minutes. An incision of 1 cm in length was made on the anteriolateral surface of each tibia and humerus and the soft tissue was reflected to expose the broadest area of the diaphysis. A bone trephine with an O.D. of 2.5 mm and sterile water coolant water were used to prepare the bony

defects in the tibias and the humeri. Each hole extended through the cortical plate and into the medullary cavity. The proteolipid, in the form of a viscous paste, was inserted into the tibial defects. The wound sites in the humeri were left void. All surgical wounds were tightly sutured and the animals were returned to their individually marked cages.

At 7-day intervals for 28 days, 5 rats were sacrificed by administration of an overdose of sodium pentobarbital. The experimental and control sites were evaluated by gross visual inspection after the surgical removal of overlying soft tissue. A bone saw was used to retrieve the experimental and control wound areas from the host bone. The specimens were fixed in 10% buffered formalin and then decalcified in Bankuthy's medium. The decalcified specimens were sectioned at 6 microns and were stained with hematoxylin and eosin. Prepared slides were evaluated with a Zeiss Videoplan Image Analysis System with Osteoplan™ for quantitative bone morphometrics.

RESULTS

Gross Appearance

By 7 days the experimental sites filled with the lysozyme-acidic phospholipid complex (proteolipid) appeared to have a bony callus formation sealing the wound bed. The control areas appeared to possess a connective tissue plug. By 14 days it was evident that hard tissue had obliterated the prepared bony cavities in the tibias that were treated with the proteolipid. A hard tissue callus was apparently present in three out of five of the controls, while in a fourth area, soft tissue speckled with hard deposits was evident. In the fifth control wound complete soft tissue union had occurred. All proteolipid treated bony defects were completely filled with a hard tissue mass by the 21 and 28 day levels. Grossly, this hard tissue of the wound site was barely distinguishable from the contiguous host bone. At

21 days the control areas all appeared to have healed by a bony union. At 28 days the control sites had an appearance similar to the experimental sites.

Histological Appearance

The histologic appearances of the control and experimental wound areas essentially confirmed the gross examination. The experimental zones demonstrated osseous bridging by 7 days (FIG 1). There was some amorphous proteolipid present in the 7 day specimens within the medullary cavity (FIG 1). The control defects at 7 days displayed loosely organized connective tissue without the presence of any new bone formation. At 14 days the proteolipid promoted wound repair was seen as a generalized robust osseous response (FIG 2). Numerous well-formed trabeculae with plump rimming osteoblasts were frequent, and new osteoid was abundant (FIG 3). In the control areas, by 14 days a bony bridge was evident in three out of five sites (FIG 4). By 21 days the control areas demonstrated more numerous trabecular healing than at 14 days (FIG 5). By 21 and 28 days the experimental cavities that had been filled with proteolipid were virtually repaired by osseous regrowth (FIG 6 and 7). The control defects, by 28 days, also appeared to be completely obliterated by new bone; the exception was one specimen where a connective tissue plug had been generated (FIG 8).

DISCUSSION

Acidic phospholipids have been shown to be highly effective binders of calcium ions; however, calcium ion binding is not tantamount to mineral formation.[8] Perhaps it is the subtly intricate and delicate relationship between the acidic phospholipid and the extremely basic protein, lysozyme, that establishes the environment suitable for nucleation and calcification. In this regard, it has been established that the initiator of dental calculus matrix calcification, *in vitro*, is an acidic phospholipid complexed

with a protein (a proteolipid).[7] Indeed, proteolipid has also been shown to be a nucleator of bone matrix calcification, *in vitro*.[6]

Ultrastructural studies of various tissues suggest that initial calcification is related to the presence of extracellular matrix vesicles.[9] These membrane-bound structures are essentially protein and an acidic phospholipid.[10] Work accomplished by several investigators indicated that calcifiable complexes could be created by conjugating different basic proteins with acidic phospholipids.[11] The requirements for the process of calcification of these complexes consists of the ability to establish insolubility in an aqueous environment; the capacity to develop stable, membrane-like aggregates; and the presence of a sufficient number of acidic phospholipid sites for the binding of calcium. It may be hypothesized that bilayers are established by the synthetically contrived complexes of protein (lysozyme) and acidic phospholipid (diphosphoinositide). This structural unit is singularly important for the orchestration of nucleation and calcification. It has been suggested that the protein's function in the unit structure is twofold: (1) the development of a stabilized acidic phospholipid domain, and (2) the development of a spacer molecule to mitigate against the lipid bilayers becoming cemented together during the calcium binding.[12] Once calcium becomes bound to the acidic phospholipid, the environment is established for the formation of cluster growth of $Ca_9(PO_4)_6$.[12] The growth of $Ca_9(PO_4)_6$ at each bound calcium, forms a series of end-to-end units, which results in neoapatite formation.[12] That such an alignment of the unit structure can occur was interpreted from ultrastructural studies with the calcifying lysozyme-diphosphoinositide complex, *in vitro*.[12]

From the data collected in this investigation, the calcification ability of the lysozyme-diphosphoinositide complex *in vivo* appears to offer promise for the repair of osseous defects. According to the parameters of osseous healing we established while employing the Zeiss Videoplan Image Analysis System with

Videoplan™, the quantitation of bone morphometrics of the rate of osseous healing in the wounds treated with the lysozyme-diphosphoinositide complex was considerably more rapid than the control defects. The lysozyme-diphosphoinositide complex apparently established an environmental milieu favorable for nucleation and calcification to proceed at an accelerated tempo. Importantly, the proteolipid complex did not impede the osseous healing. In time, at the 21 to 28 day levels, the control wounds were able to approach the experimental sites in terms of healing rate ($p<0.005$). The application of an osteogenic inducing agent that can hasten bony wound healing has considerably provocative appeal. If the healing of bony wounds can be successfully and consistently accelerated, convalescence can be markedly reduced and quick recovery will be realized.

CONCLUSION

The synthetically prepared proteolipid complex (lysozyme-acidic phospholipid) demonstrated the ability to accelerate the rate at which an experimentally induced osseous wound would heal by bony union.

MILITARY DISCLAIMER

Commercial materials and equipment are identified in this report to specify the investigative procedures. Such identification does not imply recommendation or endorsement or that the materials and equipment are necessarily the best available for the purpose. Furthermore, the opinions expressed herein are those of the author and are not to be construed as those of the U. S. Army Medical Department.

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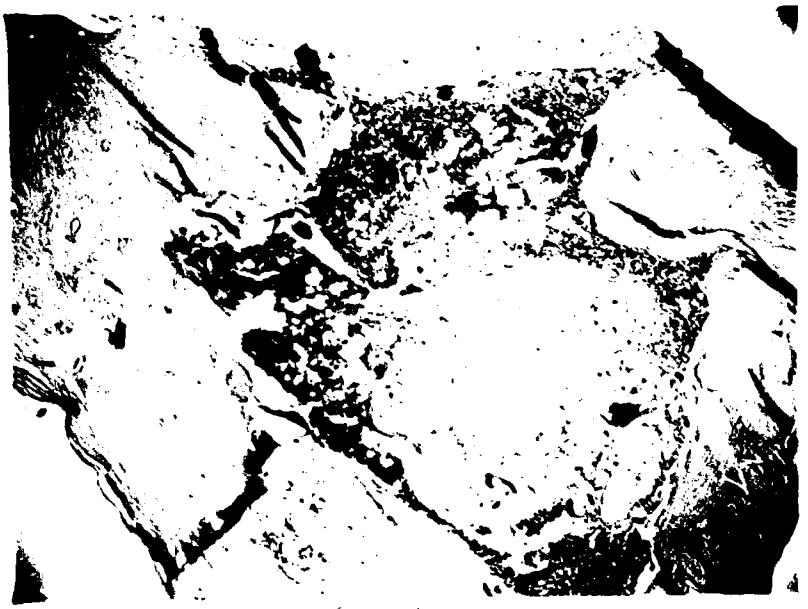

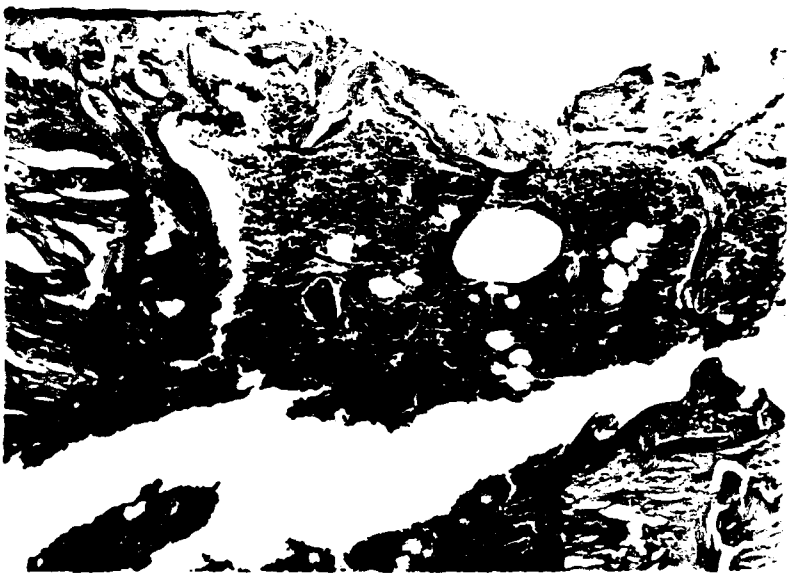

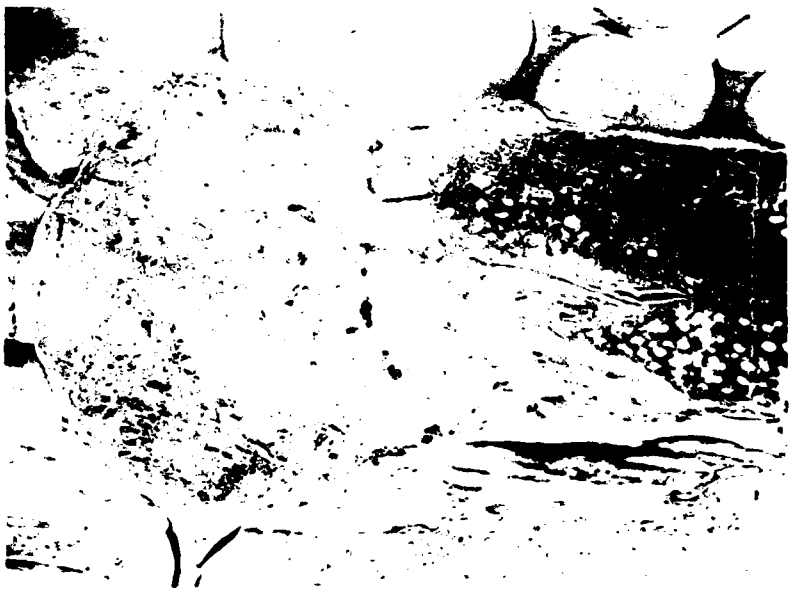

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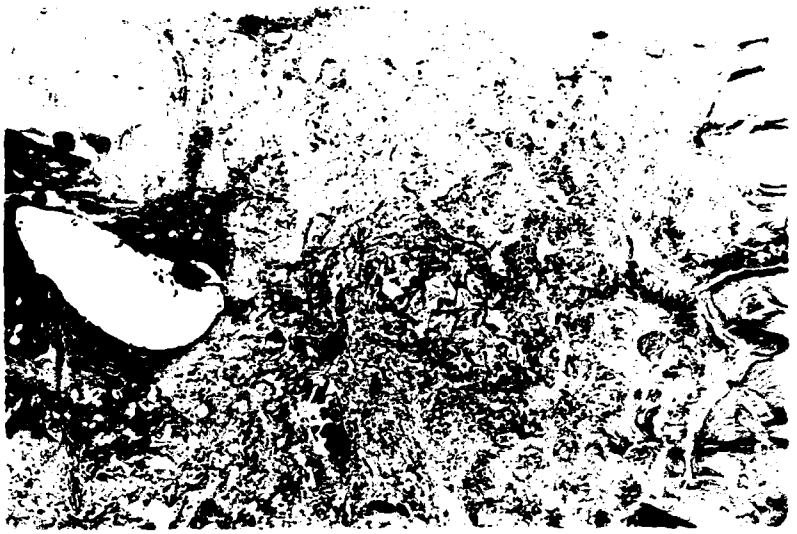

FIG. 8


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