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THE PRESENCE OF IRON IN OSTEOID SEAMS AND DENTINAL TUBULES

A. R. VILLANUEVA, B.S., M. KELIN, M.D., B. N. EPKER AND H. M. FROST, M.D.

INTRODUCTION

The presence of Prussian blue demonstrable iron in tissues has been known since the last century. Its demonstration in bone followed shortly after its discovery in other tissues, and there were many reports and studies of its presence in bone around the turn of the century.\textsuperscript{1,2} It was most often observed in the bones of children with vitamin D deficiency rickets.\textsuperscript{2,3} Shortly after the turn of the century, the demonstration of iron in bone fell into some disfavor as it was learned that in many instances artifact had been introduced inadvertently as a result of the great sensitivity of the Prussian blue test.\textsuperscript{4} Most of the available literature on the subject deals with the presence of iron in fibrous bone, usually that involved in the enchondral ossification apparatus or in fracture healing. There is very little information available concerning the presence of iron in human lamellar bone.

In studying mineralized bone sections from patients with various kinds of osteomalacias, we noted that the osteoid seams of an occasional case contained Prussian blue demonstrable iron. This was different in distribution from particulate, hemosiderin bound iron which can usually be found in some hematopoietic cells in the bone marrow.

In following up his observation of iron in the occasional osteomalacic, we utilized control rib sections from non-osseomalacic individuals obtained at autopsy and thoracotomy. A positive Prussian blue test occurred with one of the controls, and on checking the medical record of this person, he was found to be a diabetic. The presence of nonparticulate, Prussian blue demonstrable iron was found to be precisely localized within the osteoid seams. This was a phenomenon which we had previously noted only in an occasional patient with osteomalacia. Rechecking the medical records revealed that the previously noted iron positive osteomalacies were also known diabetics. These four people are not included in this study. See Figure 1.

Thus, serendipity led to the present study in which we report the presence of Prussian blue demonstrable iron in the osteoid seams of patients with diabetes mellitus.
Figure 1

The triple India Ink marks bracket osteoid seams in a nondiabetic patient. Mineralized cross section of rib, 125 X, Perls' ferric iron after Lison and Bunting. Wratten 8 filter. There is no Prussian blue demonstrable iron in this seam. The black bodies in the Haversian canal are granules of carborundum from the sandpaper with which the sections are ground. The quadruple lines bracket the zone of demarcation (i.e., mineralization front) mentioned in the text.

Materials

The bone examined was the middle third of the 5th, 6th or 7th rib taken from both normal and diabetic patients. The presence of iron was tested for in 70 ribs and 15 teeth from 85 metabolically normal people whose ages ranged from 15 to 90. They were approximately equally divided as to sex. The ribs were obtained in one third of the cases at elective thoracotomy for indications such as repair of hiatus hernia, patent ductus, aortic coarctation or pulmonary biopsy. Two-thirds of the cases were obtained at autopsy, death being sudden and caused by trauma, suicide, homicide, acute vascular incidents and acute asphyxiation.*

Twenty-three ribs were examined from patients with known diabetes mellitus. Most of them were being managed at the time of skeletal sampling with insulin or tolbutamide, but a few were being managed with diet alone. Two-thirds of these cases were obtained at autopsy for sudden death, causes including, in addition to those already listed, fatal infections and cardiac arrests. One-third were obtained at thoracotomy, for the indications previously listed. Thirteen of these patients would be considered healthy and metabolically normal at the time of skeletal sampling with the exception of their diabetes, which was considered under good control. The other 10 had various associated illnesses including bronchopneumonia, heart disease, gangrene and uremia.

*We wish to thank Drs. E. S. Zawadski and R. Horn for their generosity in making the material available and for allowing us access to the autopsy and clinical records of the cases.
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Six teeth were obtained from the dental clinic at this hospital, extracted from healthy diabetic patients who were under out-patient dental care. An additional 15 teeth were obtained from non-diabetic patients at the same source. We are indebted to Dr. F. Henney and Dr. William Via for the dental material.

METHODS

The fresh ribs from the autopsy or operating room were stripped of their periosteum. Mineralized sections 2-3 mm thick of both bone and teeth were sawed perpendicularly to the longitudinal axes of these structures, and then ground under running water to 50-70 microns in thickness by Frost’s method. They were then washed with dilute commercial detergent (Lux) for five minutes and rinsed with distilled water. The bone was stained for iron by the procedure of Lison and Bunting (2 per cent $K_2[Fe(CN)]_3 \cdot 3H_2O$ and 2 per cent $HCl$, which was freshly made before use). The bone is stained in this solution with gentle agitation for 20 minutes. (Note: steel and iron alloy instruments should be avoided, glassware must be chemically clean and chemicals must be of reagent grade). In positive cases, the sites containing iron are blue. In the 19 iron positive diabetic bones the blue color appeared consistently within 10 minutes. The teeth were allowed to stand 24 hours in order to allow diffusion of the reagents into the dentinal tubules.

In tabulating the findings (See Table I), one cross identifies bones where the blue appeared only in the osteoid seam or dentinal tubules. Two crosses mean it appeared in both the seams and in the marrow tissues. Three crosses mean it appeared in the seams,

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</table>

Six teeth...

The 29 cases in this report are listed. The fourth column lists the numbers of osteoid seams (O.S.) observed in those sections stained for iron. The fifth column shows the degree of osteoid seam involvement and is indicated as follows: (−) negative; (+) iron in seams or dentinal tubules only; (+++) iron in seams plus marrow cells; (++++) iron in seams, marrow cells and on endosteal surfaces; (++++) iron in seams, marrow cells, endosteal surfaces and areas of incompletely mineralized bone. A large number of sections were made of the 1st, 7th, 11th, and 16th cases during some of the procedures done to evaluate various aspects of the method. There were no seams in the sections of the 10th or 21st cases (O), so they are not rated as negative. The seams of the 13th and 17th cases were negative.

The teeth are listed in the bottom row.

In addition to these 29 cases, bones from four additional diabetics were available but had been stored in ethanol for months. They gave a diffuse and pronouncedly positive Prussian blue reaction to such an extent that their seams could not be evaluated accurately. The source of the iron in these cases must have been the tissue elements in the marrow spaces, since the material was stored in plastic capped, glass bottles.
on the endosteal surfaces and in some additional place in the bone. Four crosses mean the
iron occurred also in low density bone. Table I lists the findings in the 24 diabetic cases.

In order to be sure that artifact was excluded, each diabetic section was stained with
one to three control rib or tooth sections taken from metabolically normal people on the
same day. Under these circumstances the blue only appeared in osteoid seams or dentinal
tubules of the diabetic patients. Sections of some of the positive cases were demineralized
with HCl (some completely, some partially) to see if the iron was deposited in, or present
in, the mineralized parts of the organic matrix. It appeared only in the parts of the matrix
which had never yet been mineralized, i.e., osteoid seams, and the walls of dentinal tubules.
Sections were tested for iron transferred from the saw blades, both before and after hand
grinding. Sections prepared in distilled water were compared with sections prepared under
running tap water and with sections stored in 50 per cent ethanol for periods of time extend-
ing from an hour to two years.

RESULTS AND DISCUSSION

We found nonparticulate, homogeneously dispersed iron accurately localized to
and in the osteoid seams, and both nonparticulate and particulate iron in the dentinal
tubules, of 25 out of 29 patients with diabetes. It was present in the osteoid seams
of 19 of the 23 diabetic ribs, and in the dentinal tubules of six out of 6 diabetic
teeth, that have been tested for it at this writing. It was missing in four ribs. However,
two of these ribs had no seams in which to test for its presence. It was absent in
all 85 controls.

The iron with which this article is concerned is limited to the unmineralized
osteoid. See Figure 2. Prussian blue demonstrable iron was not found in the
mineralized parts of fresh bones or teeth, whether normal or diabetic. It cannot be
demonstrated, in diabetics or nondiabetics, in the calcified matrix of wholly or
partially acid-demineralized or EDTA demineralized fresh bones or teeth. The iron
in osteoid seams is homogeneously, evenly and thoroughly dispersed throughout them,
and even with the resolution of the oil immersion objective (1.32 N.A.) it is not
particulate in nature. It is in a chemically stable form since it is not noticeably
removed (before staining) even after sections have stayed 72 hours in 2 per cent
hydrochloric or 5 per cent nitric acids, or in EDTA, or in strong oxidizing agents.

In 85 nondiabetic control persons this iron was not found in the form described
in any of the 150 sections of the 70 bones and 15 teeth tested for it. In nondiabetic
cases, we occasionally did see a thin plane of iron in the zone of demarcation of the
controls (See Figure 1) which suggests that some iron containing compound may
normally be involved in the initial mineralization of osteoid. If so, it must be
quantitatively removed when mineralization begins, because persistent attempts to
show it in the already mineralized parts of the tissues failed to do so.

In time-storage tests, no detectable migration of iron was found within three
weeks in tissue stored in 50 per cent or 70 per cent ethanol. Definite migration
occurs in tissues stored longer than this, but its pattern of staining is distinctive
and permits its recognition as artifact at a glance. Such iron appears as an intense
stain on exposed endosteal bone surfaces, and occasionally also on the walls of
osteocyte lacunae that are close to endosteal bone surfaces. This surface staining
must be distinguished clearly from the homogeneous distribution of iron in the osteoid
seams and walls of dentinal tubules that is the subject of this report. In material stored for many months the whole tissue, including the mineralized part, would often stain so intensely blue that light microscopy was impossible due to the high optical density of the stained tissue.

The necessary precautions were taken to rule out the possibility that we were seeing artifact. Although artifacts could be produced at will, they did not produce the homogeneous staining of osteoid seams described and illustrated in diabetics. An obvious source of artifactual iron is the saw blade used to cut the sections. This leaves enough iron behind to stain most of the sawed bone surfaces blue with the Prussian blue test. However, the osteoid seams do not take up this iron, which is particulate under high magnification. The iron from the saw blade seems to be removed quantitatively by grinding the sections on carborundum paper. The most convincing demonstration of the validity of this report is the reproducibility with which the iron phenomenon appears only in the diabetic in sections of bone and teeth taken from both diabetic and control subjects on the same day, and processed simultaneously in the same solutions and containers.

Figure 2
The triple marks bracket an iron positive seam, taken from the first case in Table I. Mineralized, cross section, 325 X, Wratten 8 filter. The spherical, black body in the Haversian canal is the tissue content of the canal. The osteoid seams average nine microns in thickness.
We cannot interpret the biochemical or genetic meaning of the iron in the osteoid and dentinal tubules of diabetics, and merely use this opportunity to acquaint others with it. A hitherto unsuspected biochemical disturbance in diabetics is suggested. It is possible that the iron we have seen is normally involved in and required for initial mineralization of organic matrix. This is compatible with Selye's observation of the role played by iron in calciphylaxis. If there is any substance to this suggestion, the increased amount of iron in the osteoid and dentinal tubules must be the result of a change in the balance between influx of the iron containing substance, and its subsequent efflux. We interpret our failure to demonstrate the iron in tissue that is already mineralized to mean that when mineralization begins, the iron is then quantitatively removed or displaced.

The phenomenon is highly reproducible. The test is simple. It promises to be useful in both clinical and autopsy work for detecting hitherto unknown diabetics, and for studying a new aspect of the disease. It is possible that prediabetic people may exhibit the iron phenomenon we describe, making it possible to diagnose them before they manifest overt disease. For example, one might examine the deciduous teeth.

Since we have not yet had an opportunity to search for iron in normal people, it is not possible to say whether the phenomenon is a specific diabetic disturbance, or if it occurs in other diseases as well. Its importance is that it may provide a means of detecting the presence of iron in tissue and for studying its role in mineralization.

Figure 3

Same magnification and filter as Figures 1 and 2. This photomicrograph shows the layer of iron found on the endosteal surfaces of many specimens, including normal people as well as diabetics. It appears as a very thin, dark blue coloration on the endosteal surfaces, and is bracketed between the India Ink marks. The tissue in the marrow spaces of this specimen (the 7th in Table I) is also markedly iron positive.
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teeth (when they are displaced by the adult teeth) of the children of diabetic parents. Since the form of iron we have described did not appear in tooth enamel (of ectodermal origin) but did appear in dentine (of mesodermal origin), the basic subdivision of the embryo into three layers may be involved in determining the distribution of this iron in tissue.

SUMMARY

Prussian blue demonstrable iron was found in the osteoid seams of lamellar bone, and in the dentinal tubules of teeth, in 25 of 29 diabetic subjects. It was not present in these structures in any of 85 nondiabetic control subjects. The iron is homogeneously distributed throughout the osteoid seam, and is both homogeneous and particulate in the walls of dentinal tubules, at light microscope levels of resolution. Its unique distribution suggests the existence of a previously unsuspected biochemical disorder in iron metabolism (at the cellular level) in diabetic persons.

REFERENCES
