THE EXPERIMENTAL INDUCTION OF LOCALISED SKELETAL TUBERCULOUS LESIONS AND THEIR ACCESSIBILITY TO STREPTOMYCIN

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There has been prolonged disagreement and confusion over the penetration of anti-tuberculous antibiotics and chemotherapeutic agents into skeletal tuberculous lesions. Surgical extirpation has been recommended by many workers on the supposition that drugs are unable to gain access to skeletal tuberculous abscess and necrotic bone (Wilkinson 1950, 1955, 1969; Orell 1951; Hodgson and Stock 1956; Kondo and Yamada 1957; Hodgson, Stock, Fang and Ong 1960). On the other hand, encouraging results of successful treatment of skeletal tuberculosis by modern anti-tuberculous drugs without routine operation have been reported by many workers more recently (Konstam and Konstam 1958; Konstam and Blesovsky 1962; American Thoracic Society 1963; Konstam 1963; Chofnas, Surrent and Severn 1964; Friedman 1966; Dickson 1967; Tuli, Srivastava, Varma and Sinha 1967*a* and *b*; Tuli 1969; Tuli and Kumar 1971). In the human disease many uncontrolled variables such as the site, duration, extent of the disease, pathological structure of the lesion, age and nutritional state of the patient, sensitivity of the organism and previous treatment make the evaluation of penetration of various anti-tuberculous agents extremely difficult and undependable. Felländer, Hierton and Wallmark (1952), Katayama, Itami, Oya, Tanaka and Maruno (1954) and Hever and Risko (1960) tried to determine the concentration of streptomycin in tuberculous material from human skeletal tuberculous lesions but found the results to vary widely from patient to patient. To resolve the controversy regarding the power of penetration of the anti-tuberculous drugs an experiment was prepared to promote chronic localised skeletal tuberculous lesions in guinea-pigs. The lesions thus induced were investigated regarding their accessibility to streptomycin.

METHOD AND MATERIAL

Production of the experimental lesion—The methods of Lindberg (1967) formed the basis of our techniques. Unvaccinated guinea-pigs were used because vaccinated animals have been reported by Lindberg (1967) to show that the lesion produced experimentally started resolving spontaneously by about five weeks. Animals eight to ten weeks old with an average weight of 380–460 grams procured by the Division of Laboratory Animal Medicine were used. The object of selecting younger animals was to operate and initiate development of the lesion before the distal femoral epiphysial plate had fused. The animals were fed on the guinea-pig food prepared by Allied Mills, Inc., Chicago, Illinois, supplemented with carrots, escarole or kale and water *ad lib*. The animals were kept under normal conditions in a laboratory separate from the main animal house. Twenty animals were used for preliminary investigations and another eighty-five were used for the planned study.

Mycobacterium tuberculosis†—Strain 107 of Trudeau Institute (referred to as TMC 107) was made available by the courtesy of the Division of Microbiology, Pepper Laboratory, Hospital

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of the University of Pennsylvania, in the form of a one-week-old broth culture with approximately known concentration of tubercle bacilli. The organisms were grown in Tb Broth (Difco) enriched with Dubos Medium Albumin (Difco) according to the method of Wells (1946) employing a New Brunswick Scientific Co. colloidrum, model TC4, rotating at 1 r.p.m. and modified to hold 250-millilitre flasks. This provided a suspension of predominantly single bacilli which was diluted to provide the desired concentration of organisms. Estimates of the number of viable organisms were determined by spreading 0.1-millilitre amounts of appropriate culture dilutions over the surface of plates of direct cord-reading agar (Lorian 1969) and incubating in the presence of 10 per cent CO₂ for three weeks. This indicated not only the number of colony-forming units, which was also a close estimate of the number of viable bacilli since most of the bacilli were present in the filtered broth culture as individual cells, but also indicated that the colony form of the virulent 107 strain was not changing. Gelfoam absorbable gelatin sponge, used in various surgical procedures for securing haemostasis, was used as a vehicle for inoculating the bacilli into the bone. Under sterile conditions 15 × 7 × 3 millimetre blocks of Gelfoam were cut and soaked in the broth culture of tubercle bacilli. For use on the control side similar pieces of Gelfoam were soaked in sterile broth. The approximate number of bacilli calculated on the bases of the amount of broth retained by the blocks of Gelfoam and the count of bacilli per millilitre of the broth varied between 45,000 and 90,000 tubercle bacilli per inoculum during the initial experiments. Preliminary investigations on twenty animals were carried out to calculate the dose of inoculum of tubercle bacilli optimum for the purpose of the experiment, that is, the dose of inoculum which would produce a chronic local lesion in a large per cent of animals without early death through systemic spread of the tuberculous disease. The preliminary studies revealed an excessive early mortality when the dose of the inoculum per guinea-pig was more than 45,000 tubercle bacilli. Therefore in the final planned experiments the average dose per inoculum per guinea-pig was kept between 20,000 and 40,000 bacilli.

Operative procedure—The distal metaphysis of the left femur was selected for depositing tubercle bacilli, whereas the distal metaphysis of the right femur was used as a control. On the control side Gelfoam blocks soaked in sterile broth were inserted into the bone whereas on the "treated side" (the left femur) the blocks were soaked in the mycobacterial culture in broth. The distal end of the femur was selected because it is readily accessible and the area corresponds to the region of metaphysis which happens to be a not uncommon site of tuberculosis in man. The soaked Gelfoam blocks were drained of any excessive fluid by placing them vertically on the walls of a sterile test tube for about five minutes before insertion into the bone.

The operation was performed under Nembutal sodium (Abbott) anaesthesia. On an average 10 milligrams of Nembutal were given intraperitoneally. After preparing the intended site of operation a longitudinal incision about 2 centimetres long was made on the lateral side of the thigh. The femur was readily approached between the muscles with minimal loss of blood. The margins of the incision from the skin to the bone were retracted by small self-retaining retractors. With a 2-millimetre dental burr an oblique hole was drilled manually in the region of the distal femoral metaphysis in a medio-distal direction. Care was taken to prevent penetration of the distal epiphysal plate of the femur. The block of Gelfoam prepared as above was inserted into the metaphysal region through the drill hole with the help of fine pointed forceps. The area was mopped to remove broth extruded from the Gelfoam and any blood, the wound was closed with steel wire and the animal was returned to its cage. With experience the operative time for both femora was eight to ten minutes.

Assessment of streptomycin sulphate penetration into the tuberculous osseous lesion—As the main aim of this investigation was to study the penetration of antibiotics into chronic lesions, only those animals were used in which the tuberculous lesion was more than six weeks old. A solution containing 15 milligrams of streptomycin sulphate, which corresponds to a dose of

nearly 1.5 grams in man, was injected intramuscularly as a single dose into the muscles of the right thigh. The animals were killed three hours after the streptomycin injection by a sudden blow on the head. Three specimens were collected from each animal: 1) blood from the heart; 2) infected material (pus, caseous material, granulation tissue) from the osseous tuberculous lesion; and 3) bone marrow and cancellous tissue from healthy bone. The material from the three specimens was tested by microbiological methods for the activity of streptomycin. These specimens were collected individually into weighed amounts of sterile broth contained in small vials, centrifuged and sterilised by filtration through Millipore 0.45 μ membranes. The concentration of streptomycin was determined by the standard tube-dilution method for

FIG. 1

Macroscopic appearance of femora nine weeks after operation. Control (*left*) looks practically normal; the inoculated femora (*right*) show typical tuberculous abscesses with caseation.

FIG. 2

Gross appearance of the bisected femora twelve weeks after inoculation with tubercle bacilli. Note chronic tuberculous abscesses in the metaphyseal region, and pathological fracture in the specimen on the right.

assaying antibiotics using a strain of *Salmonella typhosa* that was inhibited by 0.8 microgram/millilitre of streptomycin under the conditions of the test. The calculated concentration of streptomycin was expressed as micrograms of drug per gram of the specimen. The comparison was made of the drug levels found in the blood serum, material from normal bone and infected material from the diseased bone.

OBSERVATIONS ON THE LESIONS

Findings on the control side—The wound on the control side healed completely by the fifth day after operation and after seven days there was no swelling, rise of local temperature, ulceration or local tenderness. The control femora were dissected and removed from the thigh at one, two, three, six, nine, twelve or more weeks after the operation, were observed and were

preserved in 10 per cent buffered formalin for various investigations. Ninety-six per cent of the control femora revealed no evidence of an infective focus in the bone by radiological, macroscopic and microscopic investigations. At two weeks the drill hole was filled by bone and there was minimal thickening of the surrounding cortex. The bisected femur showed mild thickening of bone at the site of operation and presence of granulation tissue at the site

FIG. 3

Radiological appearance of four inoculated femora twelve, twelve, nine and six weeks after operation, with control (*right*).

FIG. 4

FIG. 5

Figure 4—Site of operation on control side one week after operation. Note Gelfoam without any peripheral reaction. (Haematoxylin and eosin, approximately $\times 10$.) Figure 5—Site of operation on control side nine weeks after operation. Note resorption of Gelfoam and replacement by normal bony trabeculae. (Haematoxylin and eosin, approximately $\times 12$.)

of placement of Gelfoam. After six to nine weeks the femur looked practically normal to the naked eye and radiologically (Fig. 3).

Histological appearance in representative specimens was as follows (Figs. 4 and 5): after one week the Gelfoam was invaded by granulation tissue made of capillaries, fibroblasts and polynuclear leucocytes with negligible peripheral cellular reaction. After two weeks the granulation tissue was being invaded by trabecular callus deposition especially at the periphery. At between three and six weeks the Gelfoam was markedly resorbed and replaced by trabecular

callus and the drill hole was filled with bone. After nine weeks the bone looked normal histologically and the callus was undergoing resorption.

Findings on the side inoculated with tubercle bacilli—After three to six days the leg started appearing swollen, warm, and tender in comparison with the control side. Between the fifth and tenth days the wound had broken down, to form an ulcer later. The animal looked ill and ate less. Radiologically there was practically no difference between the control and the inoculated side: both showed the presence of the drill hole and minimal bony reaction at the site of operation. Histologically the metaphysial region showed that the Gelfoam was being invaded by granulation tissue with peripheral cellular reaction (Fig. 6). Between three and six weeks after operation typical tuberculous ulcers had formed at the site of operation with serosanguineous or caseous matter discharging through them.

FIG. 6

FIG. 7

Figure 6—Site of operation on the tuberculous side one week after inoculation. Note Gelfoam surrounded by a mild peripheral reaction. (Haematoxylin and eosin, approximately $\times 9$.) Figure 7—Site of operation on the tuberculous side six weeks after inoculation. Note typical tuberculous abscess with central caseation. (Haematoxylin and eosin, approximately $\times 12$.)

Radiologically the diseased area showed a well developed lytic lesion with a varying degree of periosteal reaction and osteoporosis of the diseased bone. Histologically a fairly localised inflammatory area was observed occupying the region of the metaphysis (Fig. 7). Many cases revealed necrosis in the central portion. The infective focus in general was comprised of epithelioid cells and round cells. Between six weeks and twelve weeks all the inoculated animals clinically and radiologically showed fairly localised tuberculous osteomyelitis of the distal metaphysial region of the femur. The ulcer and the discharge looked typically tuberculous. Radiologically a lytic area of varying size delineated by a thin sclerotic margin was visible with varying degree and extent of bony reaction (Fig. 3). A few cases revealed a pathological fracture through the big abscess cavity in the metaphysial region. Macroscopically the size of the tuberculous focus varied from approximately four to eight millimetres in diameter (Figs. 1 and 2). Caseous pus was present in varying quantities in most of the cases. The peripheral part of the lesions consisted of pale friable granulation tissue. Histologically the lesions revealed more or less similar appearances typical of tuberculous foci, consisting of a central area of necrosis surrounded by a varying thickness of epithelioid cells (Fig. 8). The

infective lesion was surrounded by a thin layer of fibrous tissue with occasional giant cells in some lesions. In the inoculated femur scattered small tuberculous foci were seen in the parts of the bone marrow proximal to the site of inoculation.

In general the infected animals looked smaller and weighed less than healthy ones of the same age. The average weight of the diseased guinea-pigs at twenty weeks of age—twelve weeks after inoculation—was 500 grams as compared with the average weight of 630 grams of untreated guinea-pigs of the same lot at the corresponding age reared under identical conditions. Tubercle bacilli were observed by smear examination and by culture in all the lesions submitted for microbiological examination.

FIG. 8

Site of operation on the tuberculous side twelve weeks after inoculation. Note typical chronic tuberculous abscess with central caseation and necrosis surrounded by chronic inflammatory cells and fibrous tissue. (Haematoxylin and eosin, approximately $\times 18$.)

Development of lesions in lungs, liver, spleen, lymph nodes and "control thigh"—Two animals died after five and two after seven weeks, and seven died after thirteen weeks from generalised tuberculous disease. Of the animals that were killed nine weeks or more after the inoculation, tuberculous lesions were observed in the lungs of all, in the liver in 33 per cent and in the spleen in 9 per cent. The control thigh of three animals showed a soft-tissue abscess and infection of the underlying bone, probably because of wound contamination at the time of operation. Regional lymph nodes in the left groin and left iliac fossa were involved in all cases, with caseation in some.

OBSERVATIONS ON THE PENETRATION OF DRUGS

Bioassay of streptomycin—Material from twenty-three animals with tuberculous lesions of more than six weeks' duration was submitted for microbiological assay. This comprised twenty-three specimens each of blood, infected material from the diseased bone, and marrow and cancellous tissue from a healthy bone. In seven cases the antibiotic level could not be calculated reliably because of overwhelming secondary infection or technical difficulties.

The average concentration of streptomycin was as follows: blood=25.6 micrograms; infected material from diseased bone=18.4 micrograms; normal bone=11.7 micrograms. The highest concentration of streptomycin was found in blood in each case. The concentration was greater in infected bone than in normal bone in the case of thirteen animals; the same in two animals, and less in only one animal.

DISCUSSION

Experimental model—Many attempts have been made to reproduce localised chronic tuberculous lesions of bone. Previous investigations by other workers usually resulted in early death of the animal from widespread generalised disease, or tuberculous lesions occurred in only a small number of animals. The literature regarding successful induction of such lesions in the laboratory is sparse. More recent attempts have been by Trudel (1933) who injected tubercle bacilli into the nutrient artery/nutrient foramen of the femur in rabbits. Zappia and deBlasio (1935) attempted induction by depositing 1 milligram of tubercle bacilli directly into the tibia of guinea-pigs. Koch (1950) produced skeletal tuberculosis in unvaccinated rabbits

by depositing a small volume of human tubercle bacilli (about the size of a pin head) through a hole drilled in the distal part of femur. Little is however reported about the rate and duration of survival of animals thus treated. Hodgson, Wong and Yau (1969) attempted to produce tuberculosis of the spine by injecting tubercle bacilli into the paravertebral organs. Lindberg (1967) reported production of a localised tuberculous lesion in the distal part of the femur of guinea-pigs by insertion into the metaphysial region of a piece of Spongostan soaked in a concentrated suspension of tubercle bacilli. We have used a similar technique with success in all cases. Use of Gelfoam as a vehicle for the tuberculous inoculum assures minimal systemic dissemination and provides prolonged contact of the bacilli with the surrounding tissues, which seems to be necessary for them to become established locally. The metaphysial region of the lower end of femur seems to provide an ideal environment for initiating the lesion, as this region has a tortuous vascular arrangement, sluggish circulation and abundant reticulo-endothelial elements.

Role of trauma—The significance of trauma in the causation and localisation of skeletal tuberculosis has long been discussed. On the control side in the present experiment an osseous tuberculous lesion did not develop in 96 per cent of the animals. It is expected that some degree of bacillaemia must have followed the insertion of Gelfoam impregnated with tubercle bacilli on the treated side. The presence of bacillaemia is indicated by the observation of systemic tuberculous manifestations in distant viscera, and of early generalised tuberculosis especially in animals receiving a heavy dose of the inoculum of tubercle bacilli. Thus it is clear that something more than local trauma and bacillaemia is required for initiating a local skeletal tuberculous lesion. This is probably the reason why injection of bacteria into the bone marrow usually fails to produce local infective foci at the site of injection; presumably a large proportion of the organisms are rapidly transported into the general circulation. The use of Gelfoam impregnated with tubercle bacilli prolongs the duration of contact between the bacilli and the local tissues, thereby increasing the possibility of development of a local tuberculous lesion to 100 per cent. Localised tissue necrosis probably creates a similar condition favourable for prolonged contact of the organism with the surrounding tissues. Norden (1970) reported development of pyogenic osteomyelitis in 90 per cent of animals by making a local injection of sodium morrhuate followed by an injection of bacterial suspension. The material was injected through an 18 gauge needle inserted percutaneously into the metaphysial region. Sodium morrhuate presumably caused localised vascular thrombosis with devitalisation of the tissues and local necrosis.

Visceral tuberculous lesion—Many animals showed evidence of tuberculous involvement of lungs, liver, spleen and regional lymph nodes. All those animals that were killed nine weeks or more after treatment had lung involvement, 33 per cent had liver involvement and 9 per cent had involvement of spleen. Mostly the lesions were of generalised miliary nature. These observations prove that from a localised osseous tuberculous lesion a state of bacillaemia exists. This provides an extra proof of generalised nature of skeletal tuberculous disease in man warranting a systemic treatment rather than an exclusive focal attack.

Drug penetration—The experimental lesions produced resembled human skeletal lesions clinically, radiologically, histologically and microbiologically. In general, streptomycin assayed in the present study by microbiological methods entered tuberculous abscess in the femur readily and the concentration present was comparable to or greater than the levels present in the normal bone. Serum levels were higher in every case as compared to the levels in the bone. No previous evidence is reported for such a comparison, though Felländer and colleagues (1952), Katayama and colleagues (1954) and Hever and Risko (1960) observed various concentrations of streptomycin in the diseased material from human skeletal tuberculous lesions. The levels of streptomycin concentration found in the present work were of course produced by a single dose of the drug; the observed concentration was much higher than the concentration of streptomycin (1 microgram/millilitre) considered sufficient to have

an inhibitory effect on the human type of mycobacterium tuberculosis. In clinical practice as a rule more than one drug is administered over a length of time, and it may rationally be expected that the titre of concentration of the individual drugs would be higher and the cumulative bacteriostatic/bacteriocidal effect would be much greater than that observed with a single dose of the drug.

Lindberg (1967) demonstrated the presence of radioactive dihydrostreptomycin in necrotic regions and the abscess of skeletal tubercular foci produced experimentally in guinea-pigs. Similar observations have also been made by André (1956) and Hanngren (1964). Hanngren (1959) also observed diffusion of radioactive para-aminosalicylic acid in tuberculous abscesses. Barclay, Ebert, LeRoy, Manthei and Roth (1953) and Canetti (1955) observed by the use of radioactive tagged isoniazid, that this drug was freely diffusible into all tissues including bone, as well as into abscess cavities and even dry caseous material in sufficient concentration to destroy bacilli.

Regardless of the precise pharmacodynamics, it is clear that antituberculous drugs when administered systemically achieved concentrations in the osseous tubercular lesions in excess of the usually accepted *in vitro* inhibitory levels against the mycobacterial organisms. It may be rationally presumed that if the organism is sensitive to the antituberculous drugs and the drug is administered for sufficient length of time this may well control the infection and permit the lesion to have a natural healing. If a lesion in clinical practice does not come under control by modern antituberculous drugs the reason is not failure of the drugs to reach the lesion through defective penetration or diffusion. The cause of failure must be sought in other variables such as the nature of the mycobacterium (atypical forms being generally resistant), resistance of the infecting organism to the drugs being administered, and the mechanical nature of the pathological lesion—for instance, the presence of big sequestra.

SUMMARY

1. Chronic tuberculous osseous lesions were induced consistently in eight- to ten-week-old unvaccinated guinea-pigs by the insertion of Gelfoam impregnated with mycobacterium tuberculosis into the metaphyseal region through a drill hole in the distal part of the femur. Typical tuberculous lesions developed by three weeks and many of them were followed for twelve weeks or more.
2. This experimental model establishes a reliable method of producing a localised lesion at a predetermined site without early death of the animal. The model is sufficiently similar to the human lesion, and may offer a reliable system for further investigations.
3. It was observed that streptomycin penetrates readily into tuberculous osseous lesions. The concentration of streptomycin found in the tuberculous lesion after a single intramuscular injection was much higher than the concentration considered sufficient to have an inhibitory effect on the human type of mycobacterium tuberculosis.

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