

Evaluation of Locally Induced Osteoarthritis by the Complete and Incomplete Freund's Adjuvant in Mice. The Application of DEXA Measurements

(histology / bone absorptiometry (DEXA) / bone ashing / inflammation / mice)

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Abstract. The inflammatory reactions elicited in mice by subcutaneous injections of IFA and CFA had opposite effects when tested on local metacarpal shank bones and the distal epiphysis of shank bones. Although the intensity of the immune reactions was similar, IFA induced bone loss, while CFA induced bone formation, which was mostly periosteal in nature.

BMC and BMD measurements were assessed by means of high resolution DEXA, using a hologic 4500A bone scanner with software dedicated for the analysis of small animal bones. DEXA scans were evaluated and related to histological and bone ash content analyses. The morphological and quantitative ash weight analyses of bones exposed to the adjuvants were consistent with DEXA bone density scan measurements.

It is a common belief that the inflammatory reaction taking place in the vicinity of bones is combined with bone destruction (osteolysis). The primary goal of this paper is to imply that for some types of immune reaction bone accretion dominates over the bone resorption, and that such bone involvement could be evaluated by the dual energy X-ray absorptiometry (DEXA) method.

Inflammation and bone loss are combined in several pathological conditions, such as osteomyelitis, periodontal disease (Goodson et al., 1974; Oates et al., 1996) and rheumatoid arthritis. Inflamed nasal mucosa has been shown to stimulate bone resorption of neighbouring bones when tested in an organ culture system (Kimmam et al., 1987). Numerous cytokines released in the course of inflammatory reactions have been implicated in the pathogenesis of bone loss (Frost et al., 1997; Kawashima and Stashenko, 1999). It is postulated that the effect of cytokines IL-1 and TNF- α on bone resorption is determined by a balance between levels of nitric oxide (NO) and prostaglandin E₂ (PGE₂). High NO concentrations inhibit bone resorption by antagonizing the effect of PGE₂, whereas low NO concentrations act together with prostaglandins to enhance bone resorption (Ralston and Grabowski, 1996).

The inflammatory reaction can induce bone loss, but apart from this it can also activate bone formation and bone remodelling. Many studies have shown that non-steroid anti-inflammatory drugs (NSAIDs) inhibit bone formation during for example fracture repair (Sudmann et al., 1979) and therefore support a role for inflammation in bone repair as distinct from chronic inflammation, which promotes bone destruction.

The stimulation of osteogenesis is observed following an inflammatory reaction elicited by local administration of antigens from *Corynebacterium parvum*, bacillus Calmette-Guerin (BCG) and by plant lectins concavalin A (Con A) and phytohaemagglutinin (PHA) (Włodarski, 1989; Włodarski, 1991; Włodarski and Galus, 1992). In mice the immune reaction related to the regression of Moloney sarcoma virus (MSV) is associated with a stormy osteogenesis (Włodarski et al., 1979). Adjuvant-induced osteoarthritis in rats is associated with extensive bone erosion (Francis et al., 1972; Binderup, 1986); however, in this species there are some reports demonstrating that the adjuvant-induced arthritis produces not only bone loss, but also new bone formation (Francis et al., 1972; Fukawa et al., 1985; Tomoda et al., 1986). Clearly, in adjuvant-induced arthritis two phenomena occur simultaneously: resorption and formation in local bones.

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Abbreviations: BCG – bacillus Calmette-Guerin, BMC – bone mineral content, BMD – bone mineral density, CFA – complete Freund's adjuvant, Con A – concavalin A, DEXA – dual energy X-ray absorptiometry, IFA – incomplete Freund's adjuvant, MSV – Moloney sarcoma virus, NO – nitric oxid, NSAIDs – non-steroid antiinflammatory drugs, PGE₂ – prostaglandin E₂, PHA – phytohaemagglutinin.

As mice are a more convenient species for testing the effects of immunomodulators on bone than rats, we attempted to find out whether mice would provide a suitable model for adjuvant-induced osteoarthritis and to evaluate the local bone response in the Freund's adjuvant-induced pathology in this species. In addition, the usefulness of bone scanning (DEXA), equipped with a software package optimized for the analysis of small animal bones, and operated in the high resolution mode, was tested for evaluation of changes in murine limb bones exposed to adjuvant-induced inflammation. Bone mineral content (BMC) and bone mineral density (BMD) data derived from DEXA scanning were compared to direct bone dry mass measurements.

Material and Methods

Animals and Treatments

Throughout these experiments the animals used were 3-month-old female mice of inbred strain CFW/LI and outbred mice MIZ, bred in the vivarium of the Department of Histology, Center of Biostructure, Warsaw Medical Academy, Poland. At the age of 3 months the mice had completed their growth. As established previously, there are no strain differences in the bone response to the murine MSV-induced tumours; thus, we have neglected the significance of strain in the present study. The right foot pads of animals were injected with either 0.1 ml of complete Freund's adjuvant (CFA) or with incomplete Freund's adjuvant (IFA). The number of animals used, their division into subgroups and the results obtained are summarized in Tables 1 and 2. CFA (Sigma, St. Louis, MO) is composed of *M. tuberculosis*, heat killed and dried, suspended in paraffin oil with mannide monooleate, while incomplete adjuvant is a mixture of paraffin oil and mannide monooleate alone. The contralateral, left pad, was not injected and remained intact to avoid additional intervention. From our earlier studies we concluded that the injection of saline elicits neither an inflammatory nor a bone reaction.

Foot pad measurement

The animals were observed daily for the presence and degree of pad swelling before being sacrificed by cervical dislocation at 2, 3, 4, 5 and 6 weeks post adjuvant administration. The diameter of both pads, measured as vertical height and horizontal width at the metacarpal region, was calculated with 1.0 mm accuracy. From these values an approximate cross-sectional area was calculated and data expressed in mm².

Lymph node measurement

The popliteal lymph nodes, which drain the pad area, were excised, cleaned of adjacent fat and connective tissue and weighed immediately on an analytical balance

with an accuracy of 0.1 mg. The weight of the popliteal lymph nodes was a parameter reflecting the intensity of the local immune reaction in the foot pad.

Histology

Foot pads and popliteal lymph nodes were excised and fixed in 10% formalin. Pads were demineralized in 10% formic acid solution and processed for conventional paraffin wax histology; 8- μ m thick sections being stained with haematoxylin and eosin. Sections of lymph nodes were examined histologically after metachromatic staining using a 0.5% aqueous solution of toluidine blue for visualization of mast cells.

Bone dry mass measurement

A random selection of whole limbs were excised, fixed in formalin and stored until bone scans were performed, while for measurement of shank bone, dry mass specimens were not fixed but were hydrolyzed in 0.1 N KOH at 65°C overnight for isolation of bones. After washing out the hydrolyzed soft tissues, the paired bones were dried and weighed on an analytical balance with an accuracy of 0.1 mg. The yield or loss of shank bones in each animal was evaluated by the subtraction of the left, contralateral bone weight from that of the adjuvant-exposed right tibia + fibular weight. The bone gain or loss was expressed as a percentage of the contralateral (control) bone weight. Any differences between paired bones exceeding 3% of the control value were considered as significant. The weight differences represented either bone formation (yield of dry bone mass) or bone resorption (loss of bone mass).

DEXA measurement

Determination of bone mass and density by dual X-ray absorptiometry in the region of whole shanks was a modification of the technique described earlier (Wahner and Fogelman, 1994). The analysis was performed using a hologic-4500A fan beam X-ray bone densitometer. Calibration was performed using a small animal step phantom with the laser positioning light about 2 cm from the thinnest step of the phantom, so that the scanner collected several lines of air before encountering the thin step of the small animal step phantom. On completion of the calibration scan, the system automatically analyses the step phantom and updates the calibration record. The values of BMD (gm/cm²) and BMC (grams) of paired bones (belonging to the same animal) were quantified under uniform conditions. The ratios of right (treated) shank bone scan data to the left (control) bone scan data were calculated for both, BMC and BMD values.

The DEXA measurements for both, control and CFA/IFA limbs were performed on excised limbs fixed in neutral 10% formalin.

Table 1. Evaluation of changes in hind limbs following a single administration of IFA into the right foot pad of CFW/LL and MIZ mice. L refers to the left, contralateral control limb, R to the right, adjuvant-exposed one.

| Duration (weeks) | Number of animals | Pad size* L | Pad size* R | Popliteal lymph node (mg) L | Popliteal lymph node (mg) R | Right shank bone status** | BMC foot pad + shank bone scan*** | BMD foot pad + shank bone scan*** |
|------------------|-------------------|-------------|-------------|-----------------------------|-----------------------------|---------------------------|-----------------------------------|-----------------------------------|
| CFW/LL mice | | | | | | | | |
| 2 | 8 | 12.0 ± 2.0 | 21.6 ± 6.0 | 2.6 ± 0.5 | 9.7 ± 4.5 | 0 | | |
| 3 | 3 | 14.0 ± 1.7 | 25.3 ± 2.3 | 1.4 ± 0.4 | 32.6 ± 1.9 | -3.3 ± 2.0 | | |
| MIZ mice | | | | | | | | |
| 2 | 4 | 10.5 ± 3.0 | 27.7 ± 4.7 | 3.5 ± 1.1 | 16.1 ± 7.1 | -5 ± 2.0 | 1.22 ± 0.33 | 0.97 ± 0.00 |
| 3 | 6 | 12.0 ± 2.7 | 25.3 ± 5.6 | 2.1 ± 0.5 | 28.0 ± 2.4 | 2.3 ± 2.9 | 1.19 ± 0.43 | 0.96 ± 0.08 |
| 4-5 | 5 | 10.8 ± 2.7 | 20.8 ± 1.8 | 2.4 ± 0.8 | 5.0 ± 4.1 | 0 | | |

*expressed in mm² as the product of width x height of the pad at the metacarpal level

**the loss (-) of adjuvant-exposed dry bone mass against the contralateral bone mass, expressed in %. 0 - no changes

***ratio of adjuvant exposed : contralateral control scan for BMC and BMD

The differences for mean values between L and R are significant at P < 0.01.

Table 2. Evaluation of changes in hind limbs following a single administration of CFA into the right foot pads of CFW/LL and MIZ mice. L refers to the left, contralateral limb, R to the right, adjuvant-exposed one.

| Duration (weeks) | Number of animals | Pad size* L | Pad size* R | Popliteal lymph node (mg) L | Popliteal lymph node (mg) R | Right shank bone status** | Shank + foot pad bone scan BMC*** | Shank foot pad + bone scan BMD*** |
|------------------|-------------------|-------------|-------------|-----------------------------|-----------------------------|---------------------------|-----------------------------------|-----------------------------------|
| CFW/LL mice | | | | | | | | |
| 2 | 8 | 12.3 ± 2.5 | 30.6 ± 3.0 | 3.0 ± 0.8 | 21.0 ± 7.7 | +4.1 ± 4.0 | | |
| 3 | 8 | 13.5 ± 1.6 | 27.9 ± 2.0 | 1.7 ± 0.7 | 18.7 ± 9.3 | +3.3 ± 1.8 | | |
| MIZ mice | | | | | | | | |
| 2 | 7 | 11.6 ± 3.6 | 41.1 ± 11.0 | 3.6 ± 1.8 | 53.0 ± 22.0 | +17.3 ± 9.8 | 1.18 ± 0.23 n = 4 | 1.07 ± 0.03 n = 4 |
| 3 | 10 | 13.5 ± 1.6 | 41.9 ± 9.8 | 4.7 ± 3.0 | 51.5 ± 27.0 | +7.2 ± 3.4 | 1.80 ± 0.32 | 1.19 ± 0.03 |
| 4 | 7 | 12.0 ± 3.0 | 37.4 ± 5.0 | 3.3 ± 1.1 | 27.5 ± 8.4 | +8.1 ± 4.7 | 1.63 ± 0.35 | 1.16 ± 0.08 |
| 5 | 5 | 13.0 ± 2.7 | 35.6 ± 8.0 | 4.1 ± 2.3 | 52.0 ± 8.1 | +11.5 ± 2.9 | | |
| 6 | 6 | 15.0 ± 0.0 | 32.0 ± 4.2 | 5.2 ± 2.8 | ND | +14.1 ± 5.2 | | |

*expressed in mm² as the product of width x height of the pad at the metacarpal level

** the yield of adjuvant-exposed dry shank bone mass against the contralateral bone mass expressed in %

*** ratio of adjuvant-exposed : contralateral control scans for BMC and BMD

The differences for mean values between L and R are significant at P < 0.001.

The differences for mean values of BMD for IFA and CFA are significant at P < 0.05.

Statistical analysis

The significance of mean value differences was estimated by evaluation of P using the Student's *t*-test.

Results

Response to the IFA

An injection of IFA into the right foot pad of inbred CFW/LL and outbred MIZ strains of mice produced swelling and induration of the right pads within two–three hours, which later reached the distal part of

the shanks. This reaction lasted until the 5th week post-injection. Such a reaction was totally absent throughout the entire observation period in the left, contralateral pad. On average, the cross-sectional areas of the adjuvant-exposed pads were two times larger than those of the contralateral pads.

The weights of the popliteal lymph nodes increased rapidly within two weeks, from 2–3 mg to 10–16 mg, and reached peak values on the third week (28–32 mg), before declining in weight, but they still remained elevated even up to the fifth week post IFA administration.