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.

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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 E_2 PHA – phytohaemagglutinin.

Inflammation and bone loss are combined in several pathological conditions, such as osteomyelitis, periodontal disease (Goodson et al., 1974; Oates et al., 1996) and rheumathoid 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 postaglandin E_2 (PGE_2). High NO concentrations inhibit bone resorption by antagonizing the effect of PGE_2, 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.

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/Ll 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 mm^2.

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-μm 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/cm^2) 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 mm^2 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 mm^2 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.

The dry mass of shank bones was either reduced slightly (up to 5% of the contralateral bone weight) or remained unchanged. The ratios of shank BMC and BMD scans of IFA-treated to the contralateral control MIZ mice were below 1.0 when measured two weeks post IFA administration (1.22 ± 0.33 and 0.97 ± 0.0, respectively), while for the three-week subgroup this ratio for BMC was 1.19 ± 0.43 and for BMD was still below 1.0 (0.96 ± 0.08).

Response to CFA

The administration of CFA into the right pad of CFW/Ll and MIZ mice produced swelling, induration and redness of the injected pads within a few minutes. This reaction was maintained up to the 6th week with very slow normalization. No reaction at all was noted in the contralateral, left pads. On average, the cross-sectional areas of the CFA-treated pads were at least 2–3 times higher than those of the contralateral pads.

The weights of the right popliteal lymph nodes increased several times and this enlargement was maintained up to the 5th week. In contrast to the animals exposed to the IFA, the animals stimulated with CFA demonstrated an increase in shank bone dry mass and this was observed consistently. The yield of bone dry mass correlated roughly with the degree of lymph node enlargement. The bone mass increase was noted as early as two weeks post CFA administration and was maintained throughout the entire 6-week observation period.

The scan analysis of shank bones revealed constantly higher values for BMC and BMD in the CFA-treated limbs compared to the control limbs. On average, the ratios of the right to the left BMC and BMD were 1.18 ± 0.23 and 1.07 ± 0.03, respectively, for the two-week subgroup and 1.63 ± 0.35 and 1.16 ± 0.08 for the three-week subgroup. The animals of both groups (IFA and CFA-treated) appeared to move to the same extent.

Histological examination

The inflammatory reaction produced by IFA and CFA administration was manifested by avid infiltration of connective tissue with mononuclear cells and fibrin deposition, qualified as granulomatous inflammation. Fibrin deposits were observed on the 7th day, while epithelioid cells, foreign cells and collagen accumulation was observed on days 14–27. This pathology lessened on the 20th day for IFA and on the 43rd day for CFA. No substantial changes in metacarpal bones were observed in the IFA-treated pads, although in some areas the inflammatory cells had slightly eroded surfaces. No osteoclastic activity was detected. In contrast, the CFA-treated pads displayed an inflammatory reaction, which was associated with avid periosteal osteogenesis. As early as on the 7th day post CFA injection, a widening of the periosteum and deposition of osteoid was observed (Fig. 1). The osteoblasts were enlarged and exhibited a strong basophilia, but were often bizzare in shape (Fig. 1, 3). In some instances within the activated periosteum, chondrocytes were also present

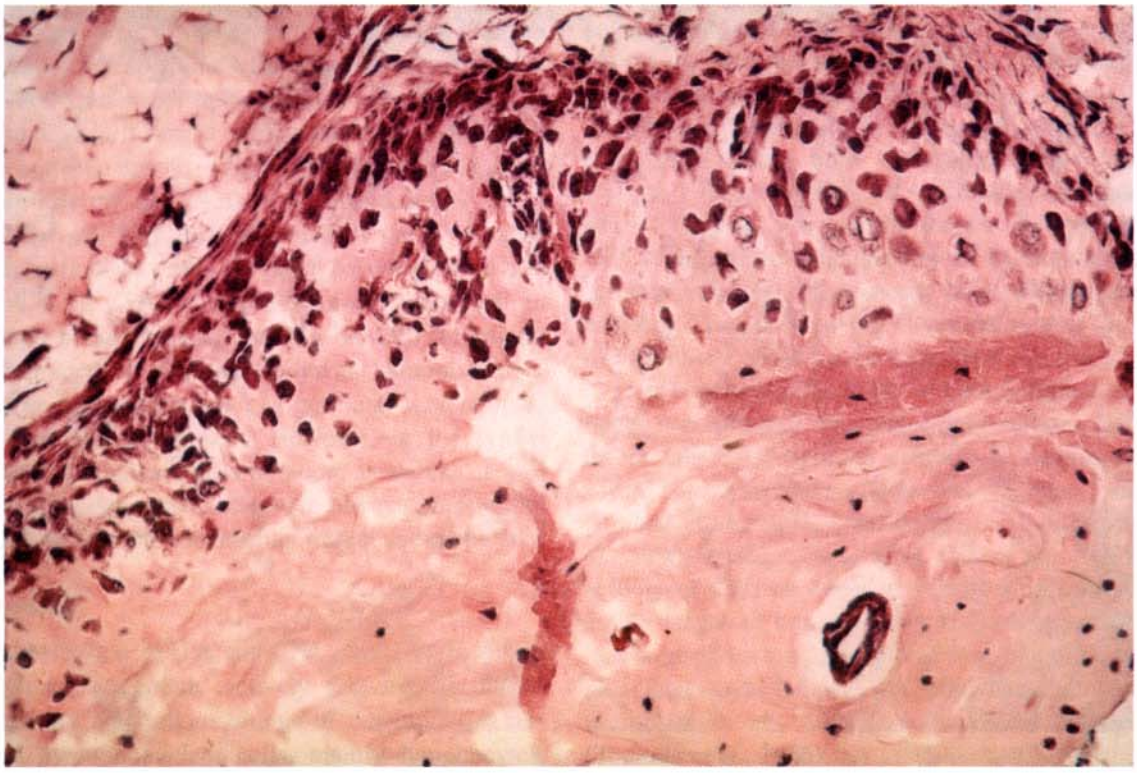

Fig. 1. Periosteal bone formation in the tarsal bone, seven days post CFA administration. Magnification 400x.

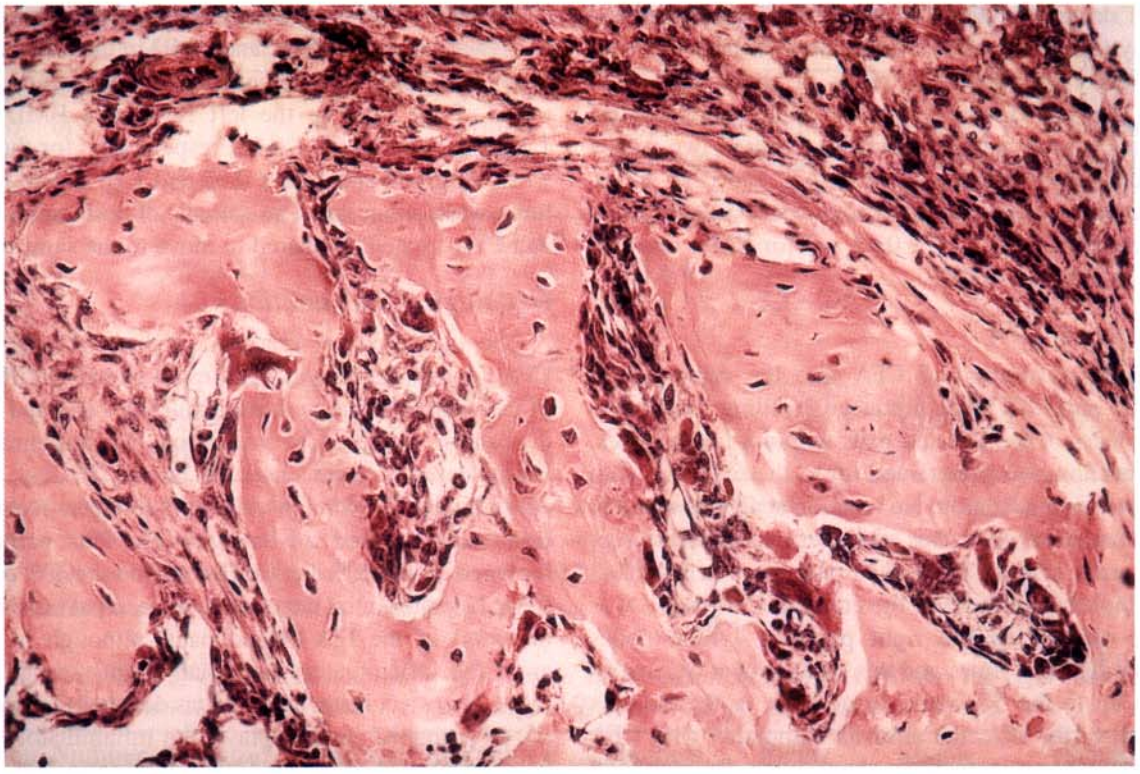

Fig. 2. Periosteal bone formation following the CFA-induced inflammation, two weeks post adjuvant administration. Bone spicules are covered with active osteoblasts; osteocytes are relatively large and entombed in spacious lacunae. Magnification 800x.

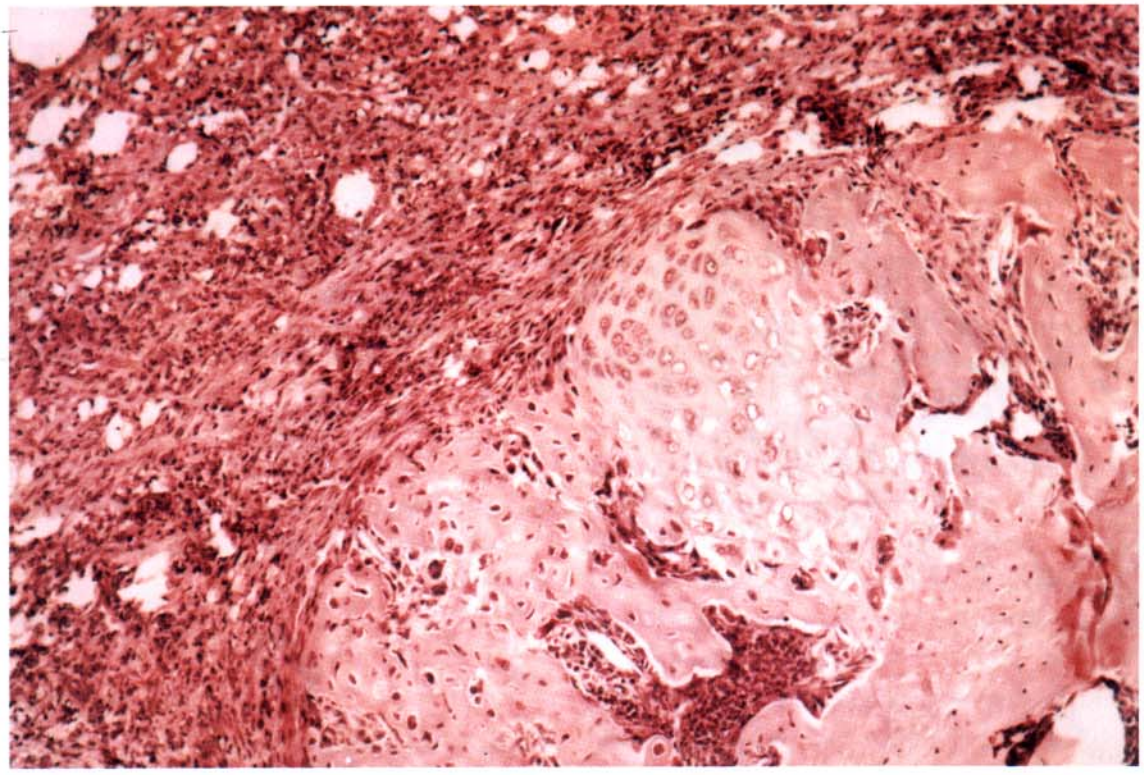

Fig. 3. Periosteal bone and cartilage formation in the tarsal bone. The inflammatory reaction evoked by CFA, two weeks post adjuvant administration. Magnification 400x.

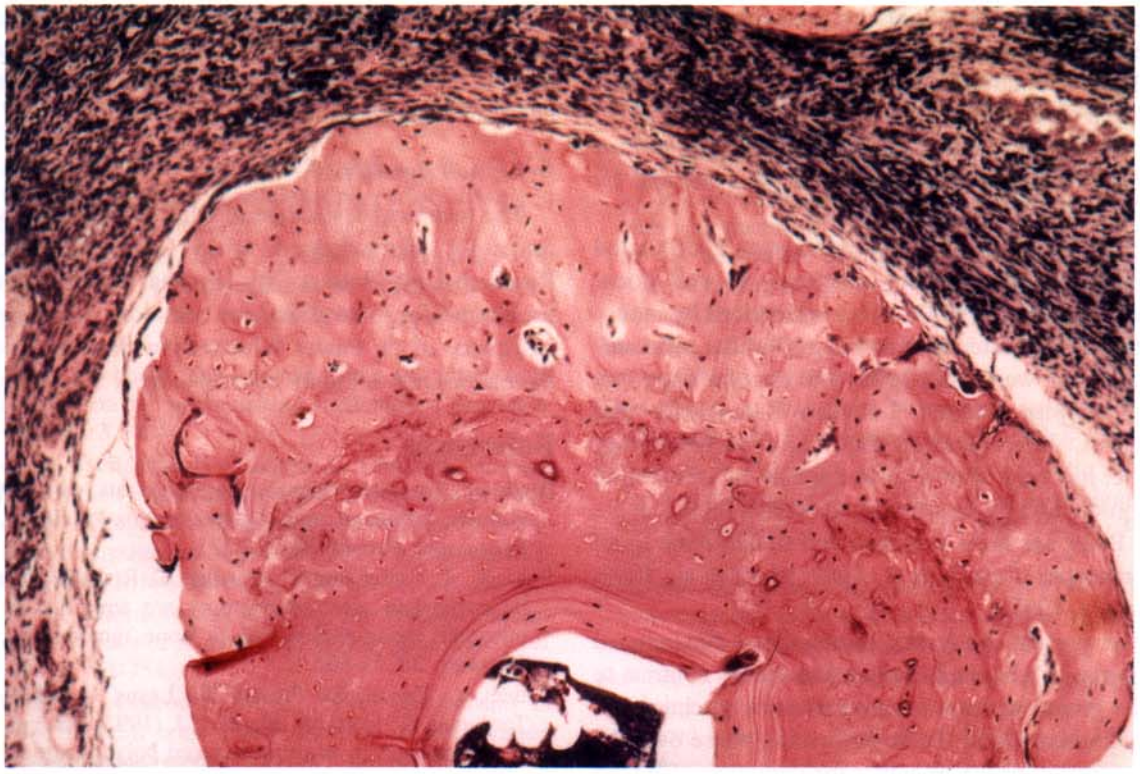

Fig. 4. Exostosis of tarsal bone, 27 days post CFA administration into the foot pad. An inflammatory reaction around the tarsal bone is still evident. Magnification 400x.

(Fig. 2). With progression of time the ossicles matured, the osteocytic lacunae, originally large, became smaller and the newly formed bone has formed a solid block together with the resident bone (Fig. 4). This newly formed bone was covered with periosteum, but no osteoclasts were apparent within it. In contrast, osteoclasts were observed frequently inside the enlarged periosteum at early stages of the pathology (7–16 days). Spaces between newly formed ossicles were colonized by bone marrow and at more advanced stages, these regions merged with the main marrow cavity. Similar changes were observed in the distal part of shank bones involved in the CFA-induced inflammatory reaction. In the lymph nodes draining the IFA-induced inflammation, examined 13 days post adjuvant administration, besides an increased number of germinal centers, mast cells were virtually absent. However, in the lymph nodes of CFA-induced inflammation, as well as in control lymph nodes, mast cells were always present and occupied all sinuses: subcapsular, radial and medullary.

Discussion

Administration of IFA and CFA induces a localized granulomatous reaction: actively growing fibroblasts and capillary buds, aggregations of macrophages surrounded by mononuclear cells, mainly lymphocytes, and foreign body type giant, multinucleated cells. So-called epithelioid cells, which are modified macrophages, are also constituents of granuloma tissue (Robins, 1974).

It was shown previously that the administration of various immunomodulators produced both, formation and resorption of local bones with variable frequency. Vaccinium antituberculosum – BCG – dissolved in the CFA stimulated mostly periosteal osteogenesis, while the administration of BCG antigens dissolved in saline stimulated mostly bone resorption (Włodarski and Galus, 1992). As the CFA contains antigens of *Mycobacterium tuberculosis*, the possibility rises that CFA alone, without the addition of BCG antigens, can activate periosteal osteogenesis. Thus, we examined the effect of CFA on bone and compared it with the effect of the vehicle alone for CFA (mineral oil, IFA).

Data concerning the effect of CFA administration on bones is conflicting. The majority of authors claim that in rats the CFA-induced arthritis is combined with severe osteolysis and an impairment of bone formation (Bonnet et al., 1993; Okazaki et al., 1998; Yoshino et al., 1998; Zhang et al., 1999). Others reported a dual activity of CFA on bone: periostitis with osteolysis at earlier stages of arthritis and periosteal new bone formation progressing to a state of near complete ankylosis at later stages (30 days post-adjuvant administration) (Jacobson et al., 1999), or endosteal osteogenesis (Tomoda et al., 1986).

Here we report an adverse effect of CFA- and IFA-induced inflammatory reactions in mice on the local

bones. While the granulomatous reaction evoked by IFA produced loss of bone or at least did not affect the net balance of bone formation and resorption, the arthritis induced by CFA had, conversely, a stimulatory effect on the local bones, as evidenced by the net bone mass increment (ashing data), histological analysis and by bone mineral densitometry (DEXA).

Stimulation of bone cells by monocytes, a substantial component of inflammatory reactions, is well documented (Peck et al., 1985). A factor produced by monocytes that affects osteoblast activity is similar or identical to IL-1 (Gowen et al., 1985) and may be important in the coupling action between osteoclasts and osteoblasts. Interleukin-1, the major inflammatory cytokine, has been shown to be capable of stimulating PGE_2 production by rat osteoblastic cells (Tatakis et al., 1988). PGE_2 has been demonstrated to add bone to all bone envelopes (Drvaric et al., 1989; Moris et al., 1990; Jee and Ma, 1997).

In the present study we found that CFA-induced arthritis can be obtained in mice and that in contrast to rats, the granulomatous reaction in mice is characterized by domination of the bone forming phase over the bone resorption phase. Whether the depletion of lymph node mast cells is in any way related to the lack of stimulation of bone formation in the IFA-treated mice remains to be elucidated. As the IFA-induced inflammation is associated with domination of bone resorption, we suggest that the *Mycobacterium tuberculosis* present in the CFA is responsible for osteoblast activation. Cell wall components of bacteria activate bone cells involved in bone remodelling (Włodarski and Galus, 1992; Blanque et al., 1998) and may reactivate adjuvant-induced arthritis in mice (Yoshino and Ohsawa, 2000). Tomoda et al. observed endosteal new bone formation and resorption in long bones of CFA-treated rats 64 days after the treatment. In our murine model system periosteal bone formation was a much earlier event than in rats.

Data concerning the effect of IFA administration on bones is scarce. In rats the application of IFA induces arthritis and together with this destruction of bones (Kleinau et al., 1994). The development of CFA-induced arthritis in rats is prevented by IFA injected 3–4 weeks before CFA challenge (Zhang et al., 1999), most likely by deviation of the T-helper cell balance of the immune response. We report here that in mice the arthritis induced by IFA is characterized by bone mineral loss. The histological and quantitative evaluation of bone involvement in the CFA- and IFA-induced arthritis in mice is consistent with the BMD scan analysis (DEXA). In the IFA-treated mice the ratio of treated to contralateral control BMD was below 1.0, which indicated a loss of or no change in the bone mass of IFA-treated limbs. When arthritis was evoked by CFA, the ratio of BMC of adjuvant-treated against contralateral control was above 1.0, demonstrating an increase in bone mass in the CFA-exposed limbs. We postulate that the BMC of murine limbs could provide a useful method for evaluation of the bone status in the course of adjuvant-induced arthritis.

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