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(54) **UTILISATION DE VECTEURS TELS QUE ADENOVIRUS ET/OU
VIRUS ADENO-ASSOCIES ET/OU RETROVIRUS ET/OU
HERPESVIRUS ET/OU LIPOSOMES ET/OU PLASMIDES
COMME VEHICULES D'INFORMATIONS GENETIQUES
RELATIVES A L'APTITUDE DES CELLULES DE
MAMMIFERES A PRODUIRE DES AGENTS POUR LE
TRAITEMENT DE PATHOLOGIES OSSEUSES**
(54) **USE OF VECTORS SUCH AS ADENOVIRUSES AND/OR ADENO
ASSOCIATED VIRUSES AND/OR RETROVIRUSES AND/OR
HERPES SIMPLEX VIRUSES AND/OR LIPOSOMES AND/OR
PLASMIDS AS A VEHICLE FOR GENETIC INFORMATION
ENABLING MAMMAL CELLS TO PRODUCE AGENTS FOR
THE TREATMENT OF BONE PATHOLOGIES**

(57) L'invention concerne l'utilisation de vecteurs tels que adénovirus et/ou virus-adéno associés et/ou rétrovirus et/ou herpèsvirus et/ou liposomes et/ou plasmides, comme agents stimulant les cellules de mammifères en vue de la production de protéines thérapeutiques, agissant comme médicaments, au niveau cellulaire, pour le traitement génétique de pathologies osseuses.

(57) Disclosed is the use of vectors such as adenoviruses and/or adeno associated viruses and/or retroviruses and/or Herpes Simplex viruses and/or liposomes and/or plasmids as agents which stimulate mammal cells in order to produce therapeutic proteins that act as medicaments on a cellular level for the genetic treatment of bone pathologies.



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(21) Internationales Aktenzeichen: PCT/EP98/06849 (22) Internationales Anmeldedatum: 29. Oktober 1998 (29.10.98) (30) Prioritätsdaten: <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">197 47 718.6</td> <td style="width: 30%;">29. Oktober 1997 (29.10.97)</td> <td style="width: 40%;">DE</td> </tr> <tr> <td>197 47 719.4</td> <td>29. Oktober 1997 (29.10.97)</td> <td>DE</td> </tr> </table> (71)(72) Anmelder und Erfinder: BALTZER, Axel, Wilhelm, August [DE/DE]; Am Strickmorgen 12, D-41464 Neuss (DE). (72) Erfinder; und (75) Erfinder/Anmelder (nur für US): LATTERMANN, Christian [DE/US]; 1416 Macon Avenue, Pittsburgh, PA 15206 (US). WHALEN, Janey, Desales [US/US]; 1416 Macon Avenue, Pittsburgh, PA 15206 (US). ROBBINS, Paul, David [US/US]; 191 Main Entrance Drive, Pittsburgh, PA 15228 (US). EVANS, Christopher, Howard [GB/US]; 111 Maple Terrace, Pittsburgh, PA 15211 (US). (74) Anwalt: SIECKMANN, Ralf; Schumannstrasse 97-99, D-40237 Düsseldorf (DE). (81) Bestimmungsstaaten: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Veröffentlicht <i>Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.</i>			197 47 718.6	29. Oktober 1997 (29.10.97)	DE	197 47 719.4	29. Oktober 1997 (29.10.97)	DE
197 47 718.6	29. Oktober 1997 (29.10.97)	DE						
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(54) Title: USE OF VECTORS SUCH AS ADENOVIRUSES AND/OR ADENO ASSOCIATED VIRUSES AND/OR RETROVIRUSES AND/OR HERPES SIMPLEX VIRUSES AND/OR LIPOSOMES AND/OR PLASMIDS AS A VEHICLE FOR GENETIC INFORMATION ENABLING MAMMAL CELLS TO PRODUCE AGENTS FOR THE TREATMENT OF BONE PATHOLOGIES (54) Bezeichnung: VERWENDUNG VON VEKTOREN WIE ADENOVIREN UND/ODER ADENO-ASSOZIIERTEN VIREN UND/ODER RETROVIREN UND/ODER HERPES-SIMPLEX-VIREN UND/ODER LIPOSOMEN UND/ODER PLASMIDEN ALS VEHIKEL FÜR GENETISCHE INFORMATION ZUR BEFÄHIGUNG VON SÄUGETIERZELLEN MITTEL, ZUR BEHANDLUNG VON KNOCHENPATHOLOGIEN ZU PRODUZIEREN (57) Abstract Disclosed is the use of vectors such as adenoviruses and/or adeno associated viruses and/or retroviruses and/or Herpes Simplex viruses and/or liposomes and/or plasmids as agents which stimulate mammal cells in order to produce therapeutic proteins that act as medicaments on a cellular level for the genetic treatment of bone pathologies. (57) Zusammenfassung Offenbart wird die Verwendung von Vektoren wie Adenoviren und/oder Adeno-assoziierten Viren und/oder Retroviren und/oder Herpes-Simplex-Viren und/oder Liposomen und/oder Plasmiden als säugerzellenstimulierendes Mittel zur Herstellung von therapeutischen, als Arzneimittel wirkenden Proteinen auf zellulärem Niveau zur genetischen Behandlung von Knochenpathologien.								

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Use of vectors such as adenoviruses and/or adeno-associated viruses and/or retroviruses and/or herpes simplex viruses and/or liposomes and/or plasmids as a vehicle for genetic information enabling mammalian cells to produce agents for the treatment of bone pathologies

The present invention relates to the use of adenoviruses and/or adeno-associated viruses and/or retroviruses and/or herpes simplex viruses and/or liposomes and/or plasmids as vectors or vehicles for genetic information which enable mammalian cells to produce, by genetic means, therapeutic proteins that act as medicaments on a cellular level for the correction of bone pathologies.

Adenoviruses are simple DNA viruses composed of double-stranded DNA and proteins, some of which viruses are pathogenic to humans, causing infections of the eyes and respiratory tract. Many species induce tumors in experimental animals which do not correspond to the natural host, or can transform cells *in vitro*. Adenoviral vectors are altered adenoviruses that have lost the ability for *in vivo* replication as well as their typical pathogenicity.

The adeno-associated virus is also known as such, and is used e. g. for labeling cells, as described in detail in WO 95/14232.

Understood by retroviruses within the context of the present invention are Class 6 RNA viruses which contain single-stranded ribonucleic acid (RNA) and the enzyme reverse transcriptase. By means of this enzyme the RNA reaching the host cell is translated into DNA in order, subsequently, together with additional repetitive sequences, to be integrated in the host genome and, as a so-called provirus, be replicated with it. Retroviruses which are used as vectors in gene

technology are replication-deficient and apathogenic with respect to their typical pathogenicity.

The object of the present invention is to provide a method of genetic treatment for bone pathologies discussed below in greater detail, a method which comprises a special agent for enabling mammalian cells to produce therapeutic proteins. The transmission of the genetic information is made possible by special vectors.

Thus, the invention relates to the use of vectors such as adenoviruses and/or adeno-associated viruses and/or retroviruses and/or herpes simplex viruses and/or liposomes and/or plasmids as agents which stimulate mammalian cells to produce therapeutic proteins acting as medicaments on a cellular level for the treatment of bone pathologies.

According to a particular embodiment of the present invention the adenoviruses are adenoviral vectors. Of the over 40 currently known human adenovirus types such as e.g. Ad2 and Ad5 these, when transformed into a vector, can be used according to the invention. They are altered so that they lose their ability for *in vivo* replication and their pathogenicity through the elimination of the essential E1 sequence. In the place of the E1 sequence and the nonessential E3 sequence, adenoviral vectors incorporate the cDNA which codes for the therapeutic genes and marker genes. On penetration of the virus into the cell, cDNA is episomally stored in the nucleus. By means of the current protein-synthesis apparatus of cells the coded substances are then produced and expressed.

According to another particular embodiment of the present invention the adeno-associated viruses are adeno-associated viral vectors. An example of these is the adeno-associated wild-type virus described in the Journal of Virology, February 1983, Vol. 45, pp. 555-564, particularly Figs. 1 to 4 and the "Materials and Methods" and "Results" sections, to which express reference is made.

Recombinant retroviruses, which are currently being used as vectors in human gene therapy experiments, all originate from the wild type of Moloney murine leukemia (MoMuLV) retrovirus. The recombinant viruses are structurally identical to the wild type of retrovirus, but carry a genetically altered genome which codes for the therapeutic genes or marker genes. Recombinant retroviruses can no longer replicate by themselves *in vivo*, but are still infectious and integrate the genome into the DNA of target cells. The pathogenic viral genes are completely eliminated.

According to another preferred embodiment the vectors are herpes simplex virus vectors. These are neurotropic human viruses which affect even mature neurons, and can, as wild-type viruses, cause a latent-type infection. Used as viral vector is e. g. the herpes simplex virus in a replication-deficient, apathogenic form. A particular advantage of the herpes simplex virus vectors is that they also integrate large foreign cDNA fragments, in particular.

According to a particular embodiment of the present invention the liposomes are liposomal vectors containing the genetic information for growth factors or cytokines or their inhibitors, and can be transmitted to mammalian cells. With regard to liposomal vectors, reference is made to the publication of Gao and Huang in Biochem. Biophys. Res. Commun., 179: 280 to 285, 1991.

According to another special embodiment of the present invention, the plasmids are those which contain the genetic information for growth factors or cytokines or their inhibitors, and can be transmitted to mammalian cells. With regard to plasmids used as vectors, reference is made to the publication of Smith, Shepherd et al., in Gene Therapy, 3: 190 to 200, 1996.

In the process described according to the invention, therapeutic proteins are produced on a cellular level, which are growth hormones, cytokines or cytokine inhibitors. Mentioned by way of example in this connection are transforming

growth factor- β (TGF- β), e. g. TGF- β 1-5; bone morphogenetic proteins (BMP), e.g. BMP-2-7; osteogenic protein-1 (OP-1), e. g. OP-1; insulin-like growth factor (IGF), e. g. IGF-1 and IGF-2; fibroblast growth factor (FGF), e. g. FGF-1 and FGF-2; platelet-derived growth factor (PDGF); tumor necrosis factor (TNF) e. g. TNF- β or TNF- α ; interleukin-6 inhibitors, macrophage colony-stimulating factor (M-CSF) inhibitors, granulocyte/macrophage colony stimulating factor (GM-CSF) inhibitors, interleukins such as interleukin-4, interleukin-10 and interleukin-13.

According to a particular embodiment of the present invention, mammalian cells are animal or human cells, particularly bone cells, bone marrow cells, connective tissue cells and /or muscle cells.

According to a particular embodiment of the present invention, the vectors leading to production of medicaments by the transfected cells are administered parenterally.

According to a particular embodiment of the present invention the vectors leading to the production of medicaments by the transfected cells are administered by the intraosseous route.

According to a particular embodiment of the present invention the vectors, which lead to the production of medicaments by the transfected cells, are administered locally at the site of the bone pathology.

According to a particular embodiment of the present invention, called *ex vivo* processes below, mammalian cells are taken from the body for further treatment, then transduced *in vitro* by means of the aforementioned vectors, cultured in a suitable medium, and autologously reimplanted, after which these mammalian cells produce, at the site of the bone pathology, the therapeutic proteins acting as medicaments.

According to a particular embodiment of the present invention the bone pathologies are osteoporosis, local or systemic bone mass loss, bone substance loss or bone structure disorders, bone fractures with bone substance loss, defect fractures or pseudoarthrosis, bone defect conditions after operations, Sudek's disease, bone substance loss after endoprosthesis loosening, periarticular osteolysis in diseases falling within the rheumatic category, e. g. rheumatoid arthritis and/or osteonecrosis.

According to a particular embodiment of the present invention it is possible to mention, as bone pathology, also the acceleration of healing of transplants, particularly ligamentous, osseous or tendinous transplants, e.g. in knee or shoulder surgery.

According to a particular embodiment of the present invention, the genetic information (cDNA) of the therapeutically effective proteins bone morphogenic protein 2-7 (BMP 2-7), transforming growth factor- β (TGF- β), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), platelet derived growth factors (PDGFs), vascular endothelial growth factor (VEGF), or the cDNA of cytokines or cytokine inhibitors, e. g. interleukin-1 receptor antagonist protein (IL-1Ra) or tumor necrosis factor- α (TNF- α) is used in order to improve the bone structure.

According to a particular embodiment of the present invention the genetic information (cDNA) for the formation of cytokine inhibitors, e. g. interleukin-1 receptor antagonist (IL-1Ra) and sTNF α R (soluble tumor necrosis factor α receptor) and interleukin-6 inhibitors or resorption-inhibiting cytokines such as interleukin-10 is used in order to reduce the bone degeneration.

The present invention will now be explained in detail with the aid of the figures.

Fig. 1 is a plot of the production of the therapeutic protein IL-1Ra per 50,000 cells after 48 h (in pg) for osteoblastic cells transduced with MFG (Samples 1-6, (values averaging 9111.5 pg) compared with non-transduced osteoblastic cells (controls 1a-3a, values around 0 pg) and retroviral LacZ transduced osteoblastic cells (controls 1b – 3b, values around 0 pg). The measurement was performed by ELISA analysis.

Fig. 2 is a plot of the spontaneous production of alkaline phosphatase (ALP) by human osteoblastic cells (HOB) (9.5 units per 1,000,000 cells), as well as the positive controls which were carried out with murine osteogenesis imperfecta stem cells (OIM) after stimulation with BMP-2 (30 units per 1,000,000 cells). The negative controls with immortalized synovial fibroblasts (HIG-82) produce no alkaline phosphatase. Nor is any ALP present in the acellular nutritive medium (= no cells).

Fig. 3 is a plot of the decrease of the dry weight of the humeri, tibiae and fibulae in mg in ovariectomized (OVX) white Balb/C mice 12 days after surgery, in comparison to sham-operated mice. By intraosseous administration of 10^9 pfu of Ad-IL-1Ra (OVX-IL-1Ra) the bone mass loss could be reduced by about 50% compared with the untreated control group (OVX-LacZ).

Fig. 4 is a plot of the expression of the marker enzyme luciferase (units/g) in white Balb/C mice in the bone tissue, muscle tissue, lymph node tissue, as well as to a slight extent and in a transient manner also in the liver after intraosseous/intramuscular transduction of the femora with 10^9 pfu Ad-Luc. Lungs and spleen are apparently not reached by the adenoviral vectors and show no enzyme expression. In bones and musculature the enzyme expression lasts for the entire 21-day period of the investigation; in the liver an expression is only detectable for 5 days, and in the draining inguinal lymph nodes for 14 days. Thus, outside the area of administration a slight and temporally very limited transgene expression can be assumed.

Fig. 5 shows the systemic detection of transgenic IRAP (interleukin-1 receptor antagonist protein) over a period of 12 days in white Balb/C mice, for testing the quality of different forms of administration of AD-IL-1Ra in systemic bone diseases. After intrafemoral administration (◆) of 10^9 pfu of Ad IL-1Ra, positive systemic IRAP levels are found for the whole 12-day study period with maximum values on Day 3 of nearly 100 pg/mL. In contrast, on intravenous administration of Ad-IL-1Ra (●), lower values down to 32 pg/mL are attained, and a maximum duration of expression of 3 days. In the negative control carried out with 10^9 pfu of the non-therapeutic marker gene Ad-LacZ (▲) no transgenic IL-1Ra level could be systemically detected.

Fig. 6 shows the amount of deoxypyridinoline crosslinks (nM) excreted in the urine after ovariectomy (OVX) and sham ovariectomy in white Balb/C mice. It is clear that after ovariectomy there is a greater urinary excretion of deoxypyridinoline crosslinks, which serve as a direct osteoclastic activity parameter.

Fig. 7 is a 50-fold enlarged view of a paraffin section of the intrafemoral cavity of Balb/C mice after intraosseous transduction with 10^9 pfu of adenoviral vectors which code for LacZ (β -galactosidase). Morphologically, lining osteoblasts and also bone marrow cells are mainly transduced. These cells are marked by arrows.

Fig. 8 is a plot of the expression of the transgenic enzyme luciferase (units/ μ L) after injection of 10^{10} pfu of Ad-Luc into the femoral defect in white New Zealand rabbits. The distribution pattern of the enzyme activity shows that after local vector administration into the bone defect the expression of the transgenes apparently takes place mainly from local tissue structures. Bones, defect/scar tissue and musculature express the transgene with high expression values (up to 70,000 units/100 μ L in muscle). No detection of transgene activity is found in the lung, spleen and contralateral musculoskeletal tissue samples (not shown

graphically). Only in the liver can a transient, weak transgene activity be detected for 5 days. The gene expression in the bone was detectable at the longest for a total of 42 days, while in the defect-filling tissue and in the musculature, gene expression was no longer detectable after 15 and 26 days, respectively.

Fig. 9 is a 50-fold enlargement of a decalcified paraffin section from the region of the femoral defect in the white New Zealand rabbit. Morphologically, not only connective tissue cells but also adipocytes and possibly stem cells are transduced. Here, too, these cells are marked by arrows.

Fig. 10 is a 20-fold enlargement of a decalcified paraffin section from the region of the edge of defect in a femoral defect model of the white New Zealand rabbit. Histomorphologically, not only lining osteoblasts indicated by arrows are transduced, but also connective tissue cells of the adjacent scar.

Fig. 11 shows, by way of an example, three X-ray pictures of the course of healing of the femoral defect in the white New Zealand rabbit 5 weeks after intralesional transduction with 2×10^{10} pfu of adenoviral vectors which code for BMP-2. Clearly visible in all three cases is a nearly complete filling of the defect chamber with mineralized bone.

Fig. 12 shows by way of example, on the basis of three X-ray pictures, the course of healing of the femoral defect in the white New Zealand rabbit 5 weeks after intralesional transduction with 2×10^{10} pfu of adenoviral vectors which code for the nontherapeutic marker gene luciferase. Distinct osseous substance defects can be seen in the defect chamber. Comparison with the course of healing after administration of therapeutic vectors (Fig. 11) testifies to a good activity of the transgenic BMP-2.

Fig. 13 shows a production of human IL-1Ra osteoblast cells following *in vitro* transduction with Ad-IRAP after a multiplicity of infection of 1000. The highest degree of expression was noted 3 weeks after the transduction. The gene expression was detectable *in vitro* for 72 days.

The present invention will now further be explained in detail by means of embodiments, in which the data given in percent always refer to percent by weight.

Application example 1

In vitro evaluation of an osteoporosis therapy with retroviral vectors on the basis of human osteoblastic cell populations.

Material and methodology.

Used as the basis of cell cultures was human spongy bone, obtained from informed patients with therapeutic radial or ulnar resection osteotomy. Cells of arthrotic femoral heads often affected by necrotic changes were difficult to cultivate and had only a short survival time *in vitro*.

Isolation of cells

After removal of the cortical parts, the remaining spongy bone was comminuted into pieces of about 1 mm³ and then cultured. Fragments of residual cortical parts were treated overnight with 0.1 % collagenase (Seromed, Berlin) in 25 cm³ culture flasks (Nunc, Denmark). Thereupon the collagen solution was eliminated and the spongiosa fragments were washed 3 times with PBS (Seromed, Berlin). In this way the connective tissue cells released by the collagen digestion were quantitatively removed. The bone fragments were then cultivated in nutrient

medium (RPMI medium 1640; GibcoBRL, Grand Island, NY) with 10 % fetal calf serum (FBS) and 1 % penicillin/streptomycin solution (GibcoBRL, Grand Island, NY). The cells were cultivated in an incubator at 37°C under 5 % carbon dioxide gas treatment. After about 16 to 20 days the cells began to populate the surface of the spongiosa and the bottom of the culture flasks. After an additional 3 weeks the cells reached confluence and, after trypsinization with 2 mL of trypsin (trypsin, 0.25 %; GibcoBRL, Grand Island, NY, USA) and appropriate analyses were divided between cell culture flasks and cell culture dishes..

Production of virus particles

The production cell lines for the retroviral particles are based on a derivative of the NIH3T3 cell line (CRIP). This cell line contains viral *env* and *gag* genes integrated by genetic engineering. They permit the production of retroviral viruses from proviruses FG-IRAP and BAG. MFG-IRAP and CRIP-BAG are described in the publication of G. Bandara et al., Proc. Natl. Acad. Sci. USA 90, 10764 to 10768 (1993) under the title "Intraarticular expression of biologically active interleukin-1-receptor-antagonist protein by *ex vivo* gene transfer." Both cell lines produce amphotropic retroviral particles, thus show a broad host specificity. MFG-IRAP carries the cDNA of the human interleukin-1 receptor antagonist (hIL-1Ra).

BAG carries the bacterial β -galactosidase gene (LacZ) and a neomycin resistance gene (neoR), which permits a selection of transfected cells. Serving as promoter for the hIL-1Ra-cDNA and LacZ is the viral long terminal repeat (LTR). The CRIP cells are cultivated in (DMEM) medium (GibcoBRL, Grand Island, NY), 10 % FBS (GibcoBRL, Grand Island, NY) and 1 % HEPES (GibcoBRL, Grand Island, NY) in an incubator at 37°C and 5 % carbon dioxide gas treatment.

The virus-containing supernatant was collected daily from confluent cultures. After filtration through a fine 0.45 µm spray filter (Sartorius ASG, Germany) the vector suspension was stored at -80 °C until use. MFG and BAG both originate from Moloney murine leukemia virus (MoMuLV, as described by P. Wehling et al. in Spine 21:931-935 (1996) and by S. Wells et al., in Gene Therapy 2:512-520 (1995)). MFG has already been used as vector in various gene-therapeutic experiments, including a human Phase-1 study in rheumatic arthritis. The two retroviruses are no longer autonomously replication-competent, which means that an independent proliferation of the retrovirus *in vivo* is not possible.

Transduction

The osteoblastic cells were dissolved out of the cell culture flasks by trypsin digestion and brought out in 12 well-plate cell culture dishes with 30,000 cells per dish. After attaining an approximately 80 % confluence the nutrient medium was removed and the cells washed twice with 3 mL of physiological sodium chloride solution. The transduction was carried out with 1 mL of viral supernatant at a virus concentration of 1×10^6 particles of retrovirus per mL of virus supernatant, and modulated with a cationic polymer, i. e. a copolymer of N, N, N', N'-tetramethyl-1,6-hexanediamine and 1,3-dibromopropane of Abbot Corporation, known under the name Polybrene®, which is marketed by Aldrich, among others, and modulated as a promoter. During the first hour of transduction the cell culture dishes were slowly centrifuged at room temperature, and [sic] at first at 500 rpm and then incubated overnight under cell culture conditions. After 48 hours the MFG-IRAP-transduced cells were collected and frozen at -80 °C for later IL-1Ra quantitation.

Detection of LacZ transfection

48 hours after transduction the cells are fixed and the LacZ expression prepared by means of X-gal staining as follows. The cells are first washed with 1 mL of PBS solution (Seromed, Germany), then fixed with Solution 1 (1 mL of 0.5 % glutaraldehyde in 49 mL of PBS (Roth GmbH, Germany) for 10 minutes at room temperature. The cell culture dishes were then washed twice for 10 minutes each with Solution 2 (1 mL of PBS solution in 1 mM magnesium chloride). Thereupon, Solution 2 was exchanged by the substrate solution (Solution 3) consisting of 1500 μ L of 5 mM $K_3Fe(CN)_6$ and 5 mM of $K_4Fe(CN)_6$, 30 μ L of 1 M $MgCl_2$, 750 μ L of 1 mg/mL 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) and 27 mL of PBS, and left overnight in the cell culture dishes. On the next morning Solution 3 was replaced with PBS and the typical blue colorations of the cells were quantitated by optical microscopy.

Quantitation of IL-1Ra expression

IL-1Ra was determined with a commercial ELISA kit (Biosource, USA) according to the manufacturer's information.

Determination of alkaline phosphatase

The alkaline phosphatase (ALP) was determined in the cell cultures in the first passage. Immortalized synovial fibroblasts (HIG-82) served as negative controls; immortalized bone marrow stem cells of osteogenesis imperfecta mice (OIM) which were previously stimulated with recombinant BMP-2 served as positive controls. All cell cultures were subjected to a triple freeze-thaw cycle at $-80^{\circ}C$ in order to disrupt the cell membranes. The burst cells were then stored at a temperature of $-80^{\circ}C$ until further analysis. The alkaline phosphatase activity was analyzed with a commercial analysis kit of Sigma Diagnostics (Dorset,

United Kingdom) according to the manufacturer's indications and determined photometrically at a wavelength of 405 nm after 1, 2 and 30 minutes, using a UV-Max instrument of Molecular Devices, USA.

Results

X-gal staining

The transduction of retro-LacZ was carried out in three cell culture dishes simultaneously. In all cell culture dishes a blue coloration of the cells was obtained by the X-gal staining. The proportion of transduced cells in the total cell count according to optical microscopic quantitation was 60 %. No selection of the transduced cells was carried out.

Gene expression

Transduction of the cells with retro-IRAP was carried out in 6 cell culture dishes. ELISA analysis of the supernatant from all 6 cells showed that the cell populations were transduced with cDNA for hIL-1Ra and that the transgene was expressed with an average of 9111.5 pg per 50,000 cells and 48 hours at a standard deviation of 522.4 pg. ELISA analysis of the supernatant of the three culture dishes with non-transduced cell cultures, as far as the analysis of the supernatant of the cells transduced with retroviral LacZ, gave negative values, as shown in Fig. 1.

Assay of alkaline phosphatase

Alkali phosphatase (ALP) is one of the first markers that is synthesized already by immature osteoblasts and during the osteoblastic maturation process. The cell populations used in this experiment spontaneously expressed alkaline

phosphatase with a mean value of 9.5 units per 1,000,000 units, at a standard deviation of 0.7 units. The immortalized synovial fibroblasts (HIG-82) serving as negative controls expressed no ALP; in contrast to the BMP-2-stimulated immortalized OIM cells, which served as positive controls. Chosen as control cell populations were OIM and HIG-82 cells, since the power of OIM cells to synthesize ALP after stimulation with rhBMP-2 ALP is well known. Because of their ability to synthesize ALP the cells were rated as osteoblastic cell population. The results of the ALP activity tests are shown in Fig. 2.

Application example 2

Use of adenoviral vectors for developing a therapy of estrogen-deficiency-induced osteoporosis in the Balb/C mouse model

The basis of these experiments was the well-known knowledge that estrogen deficiency leads to activation of osteoclasts by systemic increase of the cytokines interleukin-1 and TNFs, which, through increased osseous resorption, causes generalized bone mass loss. The theory behind the experiments evaluating an osteoporosis therapy on the Balb/C mouse model is that, as is known, ovariectomy is followed by generalized bone mass loss which reaches its maximum after about two weeks. It is to be investigated by the use of adenoviral vectors coding for IL-1Ra, to partially prevent the bone mass loss. Until now, such a genetic process for the therapy of estrogen-deficiency-induced bone mass loss has neither been described nor experimentally evaluated.

At the time of the experiments all experimental animals were 6 weeks old. A total of five different experimental series was carried out.

First the urinary excretion of deoxypyridinoline crosslinks after ovariectomy (OVX) and after sham ovariectomy was measured after 2 days. The analysis of

crosslinks was done by means of the commercial kit of Metra Biosystems Inc. (CA, USA) according to the manufacturer's indications. This experimental series was used for evaluating the effect of ovariectomy on bone resorption and for testing the suitability of this analysis for differentiation of the bone mass loss after treatment with therapeutic vectors.

Secondly, it was investigated with adenoviral vectors coding for the marker gene LacZ (Ad-LacZ) whether transduction of bone and bone marrow by adenoviral vectors is at all possible. The vectors were administered intrafemorally through a transcutaneous intracondylar access, which made it possible to administer 100 μ L of a suspension with physiological sodium chloride solution and 10^9 pfu of Ad-LacZ by the intraosseous route. Controls were carried out with the instillation of physiological sodium chloride solution without vectors. The immunohistochemical staining method (LacZ staining is described several times in this application) makes possible the colored representation of the successfully transduced cells by the intensive blue coloration of the LacZ-expressing cells.

In a third experimental series the duration of gene expression and the intracorporeal distribution of vectors after intraosseous vector administration was investigated on the Balb/C mouse model by the administration of adenoviral markers which code for the marker enzyme luciferase. Three mice each were killed on Day 2, 14 and 21 after vector administration and different tissue types in the region of the injection as well as different internal organs [were taken] for analysis of expression of the transgene. This was carried out photometrically with the Autolumat® LB953 of Berthold Co., Germany (as described in Application Example 3) for the enzyme activity of the transgenic luciferase.

Fourth, the intraosseous vector administration more commonly carried out in the experiments was compared with parenteral vector administration, in order to investigate the duration and intensity of systemic transgene levels in both procedures. Intraosseous administration of the vectors was carried out

intrafemorally, as described above, whereas the parenteral injection was carried out into the retroorbital sinus which is generously dimensioned in the mice. The systemic IL-1Ra levels were determined in the murine serum after repeated blood withdrawals from the retroorbital sinus. The blood withdrawals took place before vector administration and then on Days 1, 2, 3, 5, 9 and 12 after injection of the vectors. The IL-1Ra levels were analyzed with commercial ELISA kits according to the manufacturer's indications.

In a fifth step the effect of the adenovirally transferred therapeutically effective IL-1Ra on the ovariectomy-induced bone mass loss was investigated. For this study four groups of at least 8 mice each were made up, with ovariectomy performed in 2 groups and the mice received either the therapeutic vector Ad-IL-1Ra or the non-therapeutic marker gene vector Ad-LacZ. The two remaining groups were treated in the same way, except, instead of the ovariectomy, only a sham operation was carried out. The analysis was done by measuring the total dry weight of the non-manipulated humeri, tibiae and fibulae, in order to be able to evaluate the systemic effect of the vector administration. The dry weight was measured in mg after exarticulation and complete de-fleshing of the bones and treatment of the bones in a 90 % acetone-alcohol bath.

Results

Analysis of the urinary excretion of deoxypyridinoline crosslinks confirms that, as shown in Fig. 6, ovariectomy is followed by an increase of osteoblastic activity with increasing bone resorption. The Balb/C mouse ovariectomy model is evidently suitable for determination of the bone mass loss and for the evaluation of a gene therapy of estrogen-deficiency-induced osteoporosis.

As shown in Fig. 7, intraosseous administration of adenoviral vectors successfully transduces a number of different cell types (arrows). According to immunohistochemical X-gal staining, which permits representation of the cells

which express the transgene, osteoblasts are transduced predominantly, but so are bone-marrow cells. Since, under the effect of suitable growth factors, osteoblasts pass through maturation to osteocytes, a prolonged expression of transgenes is to be expected because of the great stability and immunologically privileged position of these cells.

The enzyme activity of luciferase after intraosseous vector administration of Ad-Luc is shown in Fig. 4. It demonstrates that the transgene expression in bone and musculature persists over the entire 21-day study period. Transgene expression in the liver (2 days) and draining lymph nodes (14 days) is transient and does not attain the level of enzyme activity in the injection area.

Comparison of the intrafemoral vector administration preferably used in this experimental series with intravenous vector administration is represented in Fig. 5. This figure shows that the intrafemoral form of administration is superior to the intravenous form of application with regard to both intensity of expression of transgenes and duration of expression.

Fig. 3 demonstrates, on the basis of the dry weights (mg) of humeri, tibiae and fibulae that the ovariectomy-induced bone mass loss, expressed here as weight of mineralized bone, can be reduced by about 50 % by the use of gene therapy with adenoviral vectors which code for IL-1Ra.

Application example 3

Material and methods

Animals

Used in the study were white female New Zealand rabbits aged more than 6 months and weighing from 4.3 to 5 kg. In all animals, surgical defects were produced in the femur in such a way that, without treatment these defects did not heal after 9 weeks.

Surgical procedure

After introduction of general anesthesia with ketamine hydrochloride (Ketaject®) (40 mg/kg/M) and xylasin (Xyla-ject®) (3 mg/kg/M) i. m., both femura of the rabbit were shaved, disinfected with isopropyl alcohol and prepared in a sterile manner. A general anesthesia was introduced with the use of Isofluran 0.8 to 1.5 % (AErrane®) supplemented with 50 to 100 % oxygen and 50 % dinitrogen oxide. Additional local anesthesia with 2 % lidocaine was applied, while the periosteum was detached from the femora, since this procedure caused movements of the rabbits even under general anesthesia. This was followed by a longitudinal preparation of the soft parts. The entire diaphysis of the femora was prepared free and the periosteum layer was completely eliminated. A 7-hole DCP plate (Synthes, Colorado, USA) was then placed above the lateral femora and fixed with 2.7 mm self-cutting screws. An exact defect of 1.3 cm was produced by means of a ball miller. In order to protect the osteosynthesis postoperatively from the enormous bending forces during "kicking" of the rabbits, 3 cerclage wires were used, to effect an additional fixation over the plate at the distal and proximal ends of the femora. To completely eliminate bone fragments and bone marrow parts, several rinsing operations were carried out with physiological sodium chloride solution. By re-fixing the musculature around the femoral defect, a defect chamber was created. All soft part layers were very carefully and accurately closed, and the skin was sutured intracutaneously. No further splint or cast was necessary.

Vectors

For the transduction of the cells, first-generation recombinant adenoviruses (E1', E 3') were used in deleted form, one of which carried the LacZ (Ad-LacZ) gene and the other (Ad-Luc) the luciferase gene. In each case the gene expression was stimulated by a human early promoter. For each defect, 0.5 mL of a suspension of 1×10^{10} particles of adenoviral vectors were injected, either in suspension with a physiological sodium chloride solution or with purified collagen gel (Vitrogen®, Collagen Corporation, Palo Alto, California). The contralateral femoral defects received wither 0.5 mL of physiological sodium chloride solution or 0.5 mL of collagen gel without virus particles.

To produce the vectors Ad-LacZ and Ad-Luc, 293 cells were propagated. This process is described by Graham et al. in J. Gen. Virol. 36:59-72 (1977). The 293 cells were in each case transfected with adenovirus for 2 hours with a multiplicity of infection (m.o.i.) of 10. The cells were harvested after they were scratched off 36 to 48 hours after the infection and resuspended in 5 mL of a 50 mM Tris-Cl (pH 7.5) and 200 mM sodium chloride. After 5 freeze-thaw cycles the viruses are purified with 2 cesium step gradients (4 °C, 30,000 rpm, 1 hour). Ad-LacZ was dialyzed against 10 % glycerol dialysis buffer (100 mM sodium chloride, 10 mM Tris-Cl, pH 7.5; 1 mM magnesium chloride, 10 % v/v glycerol), as described by Mittereder et al. in J. Virol. 70:7498-7509 (1996), and Ad-Luc was dialyzed against 3 % sucrose dialysis buffer (150 mM sodium chloride, 10 mM Tris Cl, pH 7.5; 10 mL of magnesium chloride, 3 % v/v of sucrose. Each vector was titrated through its optical density of 260 nm. Both viruses were stored until used at a concentration of 7×10^{12} particles per mL at -80 °C.

Groups, data collection and analysis

Group 1. The femoral defect was created in 4 rabbits treated with 0.5 % physiological sodium chloride solution, which received 1×10^{10} particles of Ad-

LacZ. Two rabbits were killed after two days, and the other two rabbits were killed 12 days after the operation.

Group 2. Four rabbits were treated with a mixture of 0.5 mL of collagen gel (Vitrogen®) and 1×10^{10} particles of Ad-LacZ. The rabbits were sacrificed according to the schedule of Group 1.

Bone marrow, cortical and trabecular bone, scar tissue which fills out the defect, and muscle were analyzed for gene expression by means of X-gal staining of all rabbits of Group 1 and 2. To determine whether a systemic distribution of the vectors has taken place, tissues from the spleen, liver and lung were analyzed as well as bone, bone marrow and muscle from the contralateral untreated segmental defects. All tissue parts were fixed in 10 % formaldehyde solution for 24 hours, then decalcified in 20 % EDTA, within 2 to 3 weeks at a weekly exchange of the EDTA. After embedding of the demineralized tissue in paraffin, blocks were cut and the tissue sections stained with X-gal and eosine. The transduced cells were evaluated histomorphologically by an independent pathologist.

Group 3. Eight rabbits of Group 3 were treated with 0.5 mL of a mixture of physiological sodium chloride solution and 1×10^{10} Ad-Luc particles. The rabbits were sacrificed 2, 5, 10 and 14 days after the operation. Tissue samples were taken of the trabecular and cortical bone which surrounded the defect chamber, of connective tissue which filled the defect chamber, and of muscle which surrounded the femoral defect. To verify a systemic distribution of the vectors, lung, liver and spleen samples were analyzed together with musculoskeletal samples of the non-transduced contralateral defect. All tissue samples were homogenized, 0.1 mL of physiological sodium chloride was added and the samples were subjected three times to a freeze-thaw cycle, in order to disrupt the cells. The cells were then stored at -80 °C, until measurement of the luciferase activity was carried out. After the last thawing the samples were centrifuged at

10,000rpm (5600 x g) for 5 minutes, and the supernatant solution was set aside for measurement of the luciferase activity. The luciferase activity was measured at room temperature by means of an AutoLumat® LB953 of Berthold Co., Germany, according to the manufacturer's instructions.

Results

X-gal staining

Histomorphological analysis showed that the production of Ad-LacZ which was suspended either in physiological sodium chloride solution or in collagen gel, led to local transduction of bones, bone marrow, scar tissue and callus tissue, as well as to transduction of the muscle surrounding the injection site (Figs. 9 and 10). By contrast, no LacZ cells were found in the lung, liver or spleen, nor in the contralateral femoral defect. On using collagen gel as carrier for the adenovirus, histological study of the defect and the scar tissue which filled out the opening shows a non-resorbed solid collagen structure even 12 days after the operation. From this it can be concluded that the adenovirus has been encapsulated by the gel and hence had no possibility for transduction. The histological analysis showed no evidence of a local inflammation.

Luciferase assay

Different luciferase activity contents were found in the bone and bone marrow, as well as in the defect-filling connective tissue, and in the surrounding muscle tissue at the injection site. Five days after the transduction a slight luciferase activity was noted in the liver, an activity which completely disappeared after 10 days. No luciferase activity could be found in the contralateral femur, lung and spleen (Fig. 8).

Radiological course of healing after administration of therapeutic genes

After the preliminary studies with which the detection of the transduceability of the different musculoskeletal tissue structures was performed and the duration of expression of transgenes was determined, have shown that the femoral defect model of the white New Zealand rabbit was suitable for evaluating the effect of therapeutic genes on healing of the defect, four and six rabbits each were transduced according to the same model with 1×10^7 pfu of Ad-TGF- β and 2×10^{10} pfu of Ad-BMP-2, respectively, simultaneously with four and five control rabbits of the same age, which were transduced with the non-therapeutic marker gene Ad-Luc. X-ray controls were carried out at two-week intervals after the first control investigation done one week after the operation. In the group treated with Ad-BMP-2 a distinct mineralization spur relative to the control group was visible already after 5 weeks, as shown in Figs. 11 and 12. Final radiological examination in Week 12 showed in all 6 treated rabbits a good osseous buildup of the femoral defect chamber, as against a deficient buildup ranging all the way to pseudoarthrosis, in the nontreated control group. A similar trend was also noted in the group treated with Ad-TGF- β . In comparison to the control group, the radiological course of healing showed distinctly more mineralized substance in the femoral defect chamber, but without the formation of a radiologically visible ossification in the sense of corticotrabeular maturation. This result remained unchanged up to the 18th postoperative week.

In summary, it may be concluded that Ad-BMP-2 had a stimulating effect on ossification in the femoral defect; presumably, Ad-TGF-b has a greater enhancing action on a nonspecific mineralization of the tissue without leading to ultimate maturation of the bone. A combination of the two vectors may optionally lead to a further acceleration of bone fracture healing in defect situations.

Biomechanics

12 weeks after the operation and injection of the viral vectors into the defect the six rabbits treated with Ad-BMP-2 (2×10^{10} pfu) and 5 control rabbits which received only 2×10^{10} pfu of the marker gene Ad-Luc, were sacrificed. The operated femora were extensively de-fleshed, sparing all ectopic bone bridges. This was followed by careful metal removal. In doing so, it was found that one femur of the control group healed in the form of connective-tissue pseudoarthrosis, a second femur of the control group only showed such a thin bone bridge that it broke even upon very careful preparation. Hence this femur was excluded from the evaluation. After removal of all metal the preparations were frozen at -80°C until further analysis.

The biomechanical analysis was carried out by the three-point bending procedure in order to determine the flexural stiffness and maximum bending force of the femora.

The femora were stored in the frozen state until 24 hours before the test, and then slowly thawed in the cooling chamber. Each femur was placed in the three-point bending system that assured a free bending distance of 4.5 cm. The load was applied in posterior-anterior direction, with the load transmitted at the midpoint of the free bending distance which in all cases represents approximately the midpoint of the diaphyses.

The tests were carried out with the Instron 8500 servo-hydraulic testing system (Instron Corp., Canton, MA); an Instron 2500 lbf load-uptake cell was used. The deformation of the femora was measured with an internal LVDT system. The data were recorded by means of Instron's MAX software and analyzed with the MS-Excel software program.

A maximum bend of 1 cm was applied, at a bending rate of 0.5 cm/min or 0.0833 mm/sec. The test was ended when the femora fractured.

The test group treated with Ad-BMP-2 showed a significant difference ($p = 0.036$) relative to the control group with regard to stiffness and also with regard to the force applied ($p = 0.0055$).

Individual measurements

Stiffness		Force	
Ad-BMP-2	Control	Ad-BMP-2	Control
75.6000	80.8000	195.0000	102.0000
118.0000	64.0000	131.6000	103.2000
99.6000	53.2000	171.2000	82.0000
79.6000	0.0000	227.6000	0.0000
97.6000		206.4000	
45.6000		92.4000	

The biomechanical analysis according to the three-point-bending procedure suggests that the use of the adenoviral vectors which code for BMP-2 and thus lead on a cellular level to production of the protein acting as medicament, can bring about an acceleration of bone defect healing.

Histomorphometry

As in the above-described experiments the femoral defect model of white New Zealand rabbits was used also in this case. 16 weeks after operation of the

animals and after injection of 10^7 pfu of adenoviral vectors whose cDNA codes for the potentially therapeutic TGB-beta (Ad-TGF-beta) the animals were sacrificed and prepared. Serving as control group were 4 white New Zealand rabbits which received only 10^7 pfu of a marker gene (Ad-Luc).

The pictorial analysis was carried out on the digitalized histologic section as follows. The pictures were made sharper with a 2-fold band filter in order to define the blue-colored osseous areas (Trichrome staining). Osseous areas were defined by the color-cube technique (3 x 3 pixel), after standardizing against the normal cortical bone outside the defect. The previous defect boundaries were digitally marked and the former defect area (area of interest = AOI) was analyzed for intensity of the blue coloration. Four different measurements were carried out:

1. Absolute bone mass in the AOI.
2. Mean light intensity – measurement of the intensity of coloration makes it possible to draw conclusions regarding the quality of the newly formed bone;
3. Mass of mineralized tissue relative to the size of the defect, in percent;
4. IOD (integrated optical density, corresponding to the mean optical light intensity per surface area measured.

Determined as AOIs were the following analyzed zones:

1. Total area from the distal to the proximal screw hole;
2. Bone neoformation along the removed DCP plate;

3. Bone neoformation of the corticalis opposite the removed DCP plate;
4. Sum of defect edges.

In the test group treated with Ad-TGF-beta the total mass of mineralization in the defect region was more than twice as large as in the control group ($19.5 \pm 10.7 \text{ mm}^2$ versus $9.04 \pm 4.3 \text{ mm}^2$), but not significantly different. Even more marked were the differences when the surface area and light intensity (IOD) were taken into consideration ($1754.0 \pm 906.0 \text{ mm}^2$ versus $557.7 \pm 138.0 \text{ mm}^2$). However, even in this case the difference in the t-test for unconnected samples was not significant, which is explained by the great dispersion at small case numbers.

However, the results unequivocally indicate that TGF-beta, produced and expressed on a cellular level, has a positive effect on mineralization and ossification after defect fractures. By using the technique of gene transfer with subsequent expression of the growth factor, which, as described in the preliminary experiments, represents a predominantly local process, the well-known side effects of systemic TGF-beta administration, in particular, could possibly be prevented.

December 3, 1999
S/fk 580663WO

Amended Patent Claims

1. Use of vectors, namely adenoviruses and/or adeno-associated viruses and/or retroviruses and/or herpes simplex viruses and/or liposomes and/or plasmids as agents which stimulate mammalian cells to produce therapeutic proteins acting as medicaments on a cellular level for the genetic treatment of bone pathologies, wherein the bone pathologies are selected from local or systemic bone mass loss, bone substance loss or bone structure disorders, bone fractures with bone substance loss, defect fractures, or pseudoarthrosis, bone defect conditions after operations, Sudek's disease, bone substance loss in endoprosthesis loosening, periarticular osteolysis in diseases falling within the rheumatic category, and/or osteonecrosis.
2. Use according to Claim 1, characterized in that the adenoviruses are adenoviral vectors.
3. Use according to Claim 1, characterized in that the adeno-associated viruses are adeno-associated viral vectors.
4. Use according to Claim 1, characterized in that the retroviruses are retroviral vectors.
5. Use according to Claim 1, characterized in that the liposomes are liposomal vectors which contain the genetic

information for growth factors or cytokines or their inhibitors.

6. Use according to Claim 1, characterized in that the plasmids are those which contain the genetic information for growth factors of cytokines or their inhibitors.
7. Use according to any one of Claims 1 to 6, characterized in that the therapeutic proteins are growth hormones, cytokines or cytokine inhibitors.
8. Use according to any one of Claims 1 to 5, characterized in that the mammalian cells to be stimulated are animal or human cells, particularly bone cells, bone marrow cells, connective tissue cells and/or muscle cells.
9. Use according to any one of Claims 1 to 8, characterized in that the vectors leading to the production of the medicaments by the transfected cells are administered by the parenteral route.
10. Use according to any one of Claims 1 to 8, characterized in that the vectors which lead to the production of medicaments by the transfected cells are administered by the intraosseous route.
11. Use according to any one of Claims 1 to 8, characterized in that the vectors leading to the production of the medicaments by the transfected cells is administered locally at the site of the bone pathology.
12. Use according to any one of Claims 1 to 9, characterized in that, for the treatment, mammalian cells are taken

from the body, then transfected *in vitro* by means of the aforementioned vectors, cultivated in a suitable medium and reimplanted to produce, at the site of the bone pathology, the therapeutic proteins acting as medicaments after genetic manipulation of the target cells.

13. Use according to any one of Claims 1 to 12, characterized in that the bone pathology also includes the healing of transplants, particularly ligamentous, osseous or tendinous transplants, e.g. in knee and shoulder surgery.
14. Use according to any one of Claims 1 to 13, characterized in that, to improve the bone structure, the genetic information (cDNA) of the therapeutic proteins BMP 2 - 7 and/or FGFs and/or IGFs and/or PDGFs and/or VEGF and/or TGF- β is used, or the cDNA of cytokines or cytokine inhibitors, e.g. hLL-1Ra or TNF- α inhibitors.
15. Use according to any one of claims 1 to 13, characterized in that, to reduce the bone degradation, the genetic information (cDNA) for forming cytokine inhibitors e.g. hLL-1Ra and sTNF α R and interleukin-6 inhibitors or resorption-inhibiting cytokines such as interleukin-10 are used.

Glossary to Figures

[Caption to Fig. 3]: Dry weight of humeri, fibulae and tibiae on Day 12

Probe = Sample
Zellen = Cells
Trockengewicht = Dry weight
Tag = Day
OS-muskel = Thigh muscle
Lymph-Knoten = Lymph nodes
Leber = Liver
Lunge = Lung
Milz = Spleen
Knochen = Bone
Defekt = Defect
Muskel = Muscle
pro = per

Fig. 1

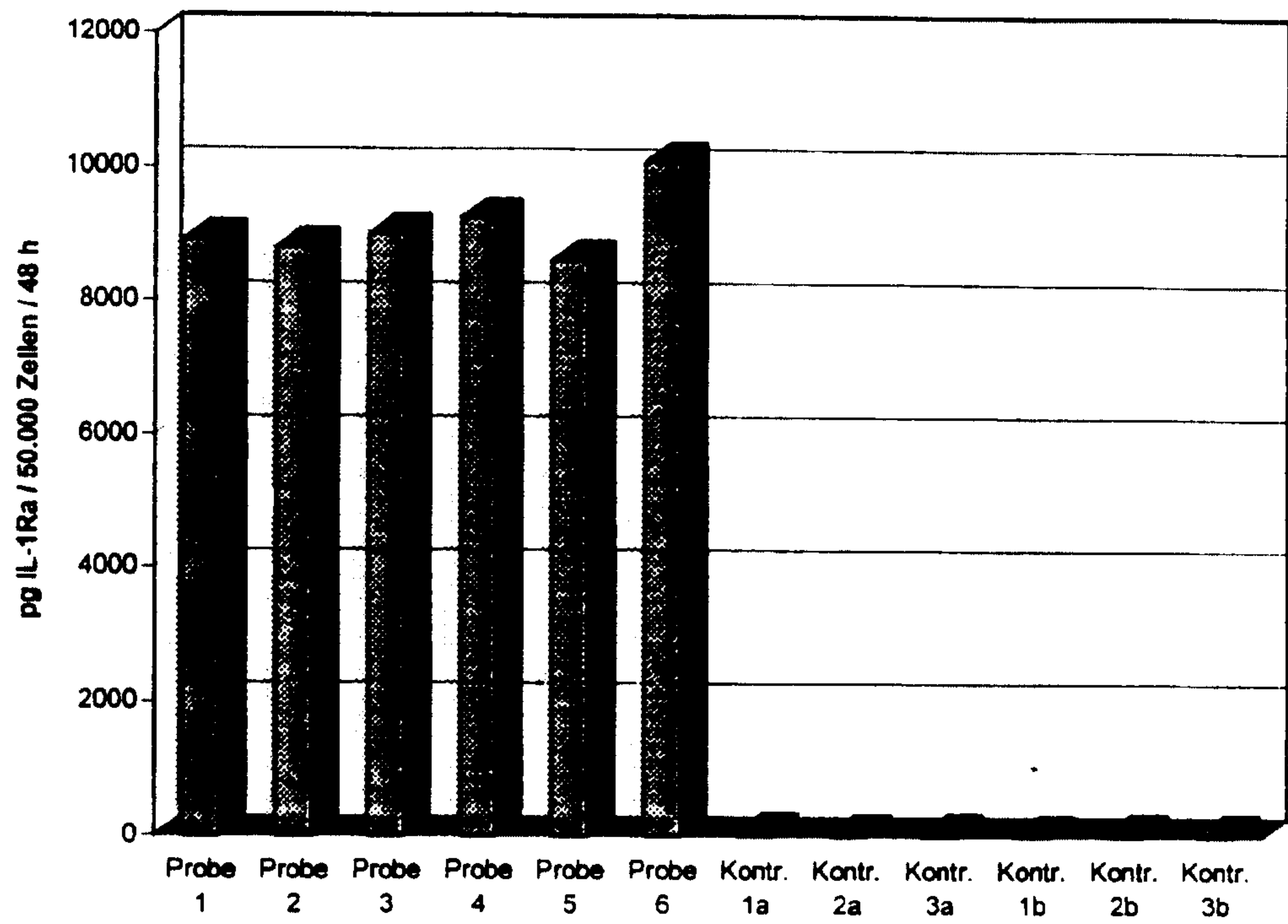


Fig. 2

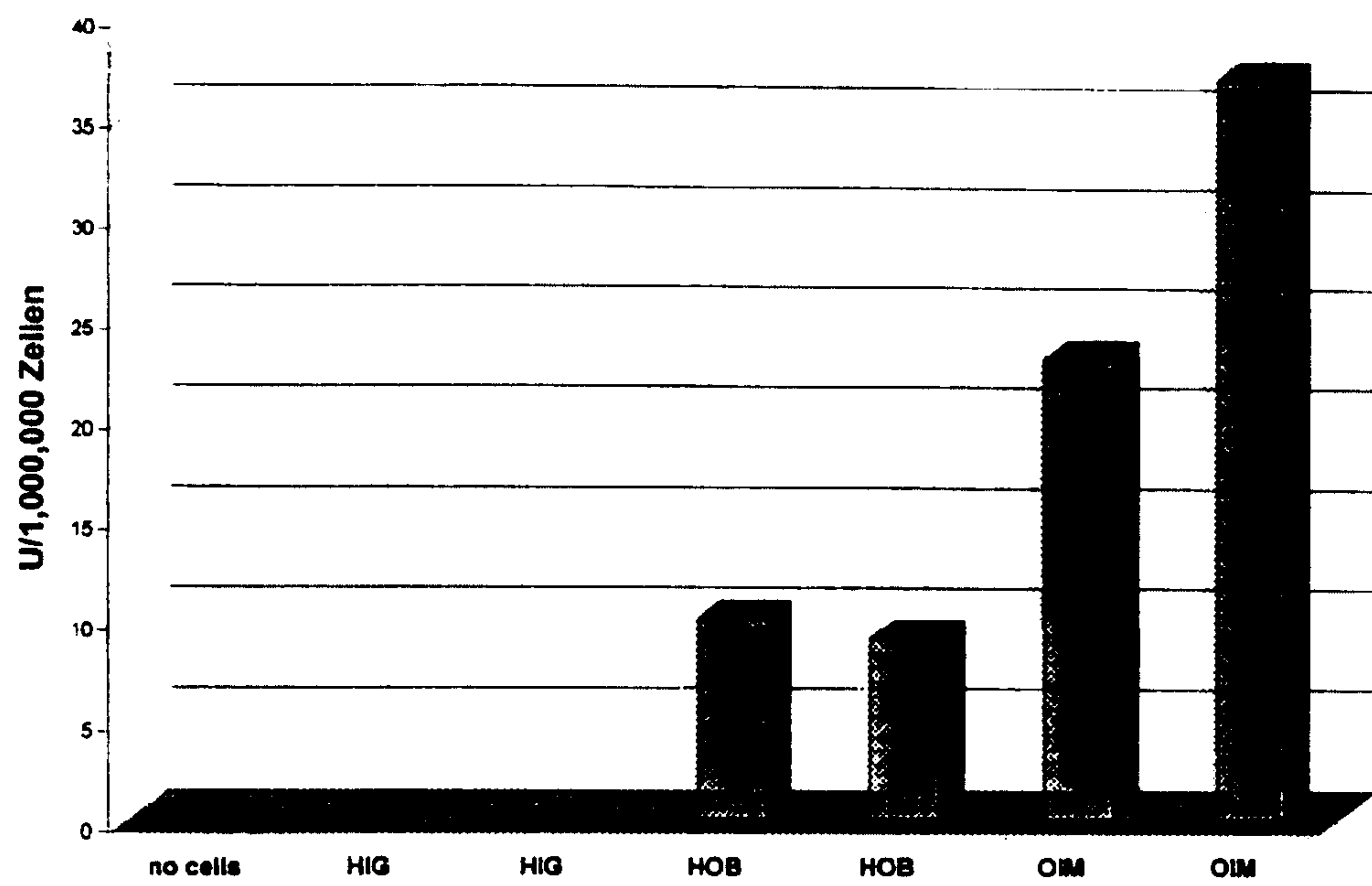


Fig. 3

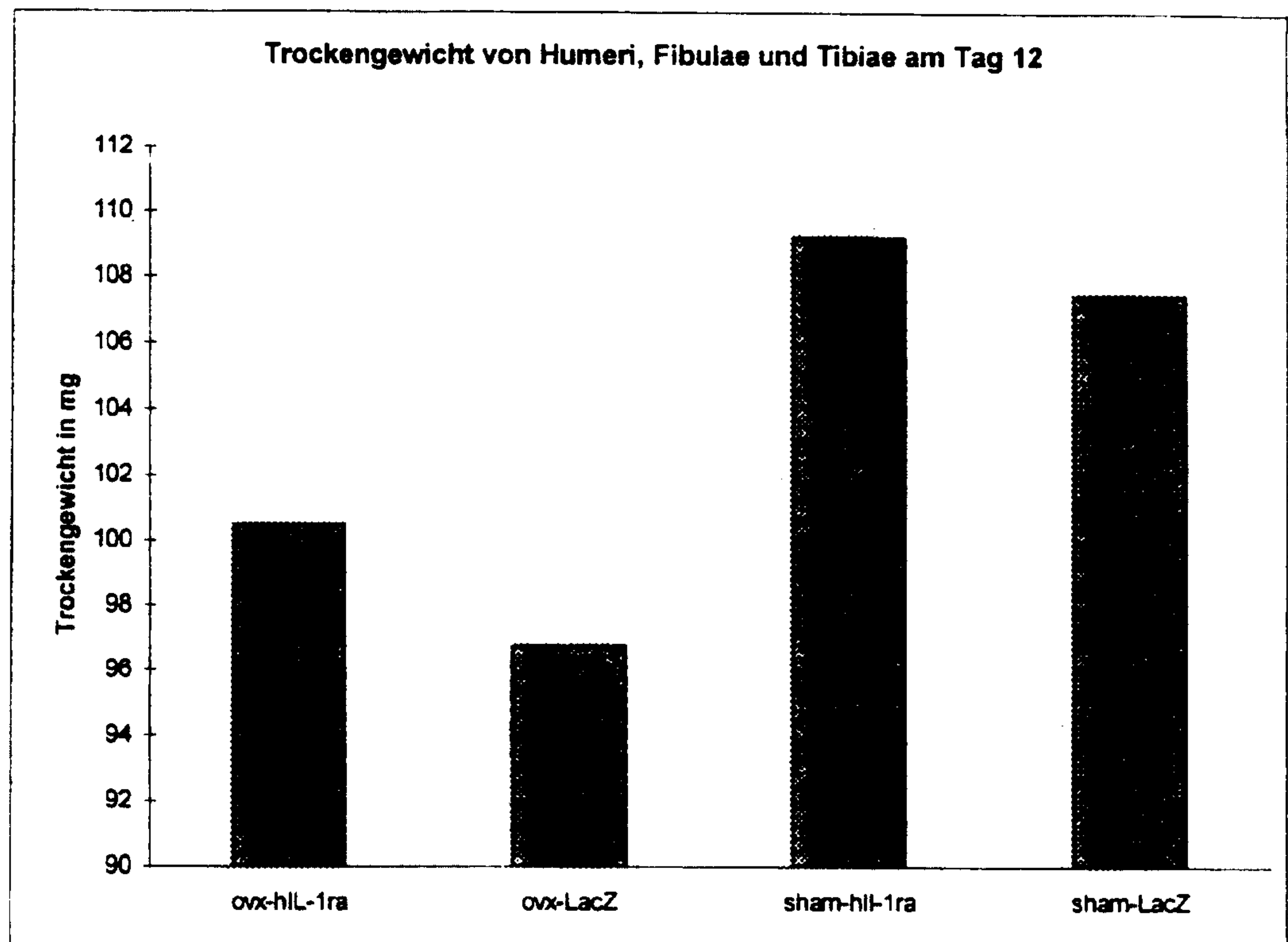


Fig. 4

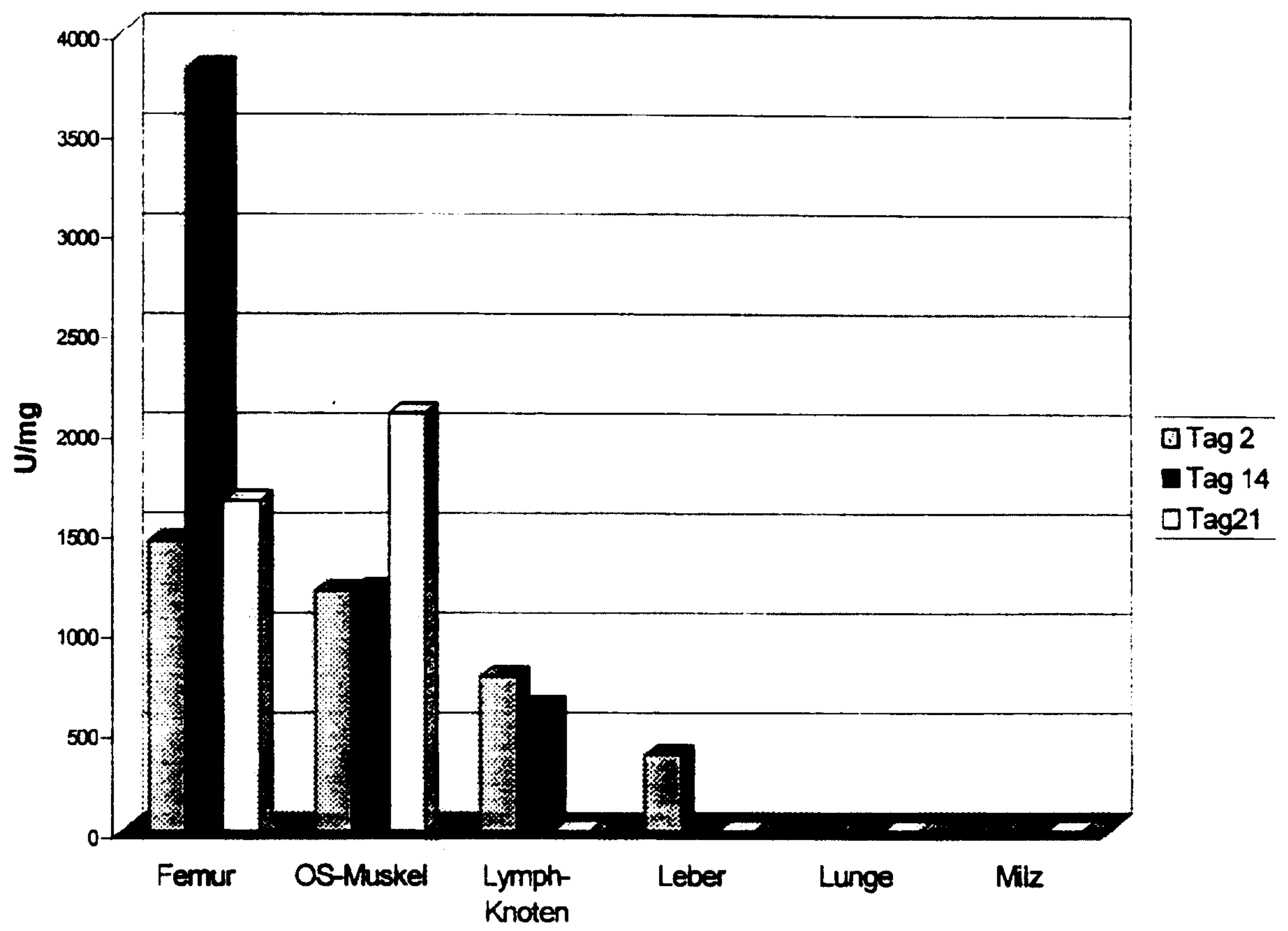
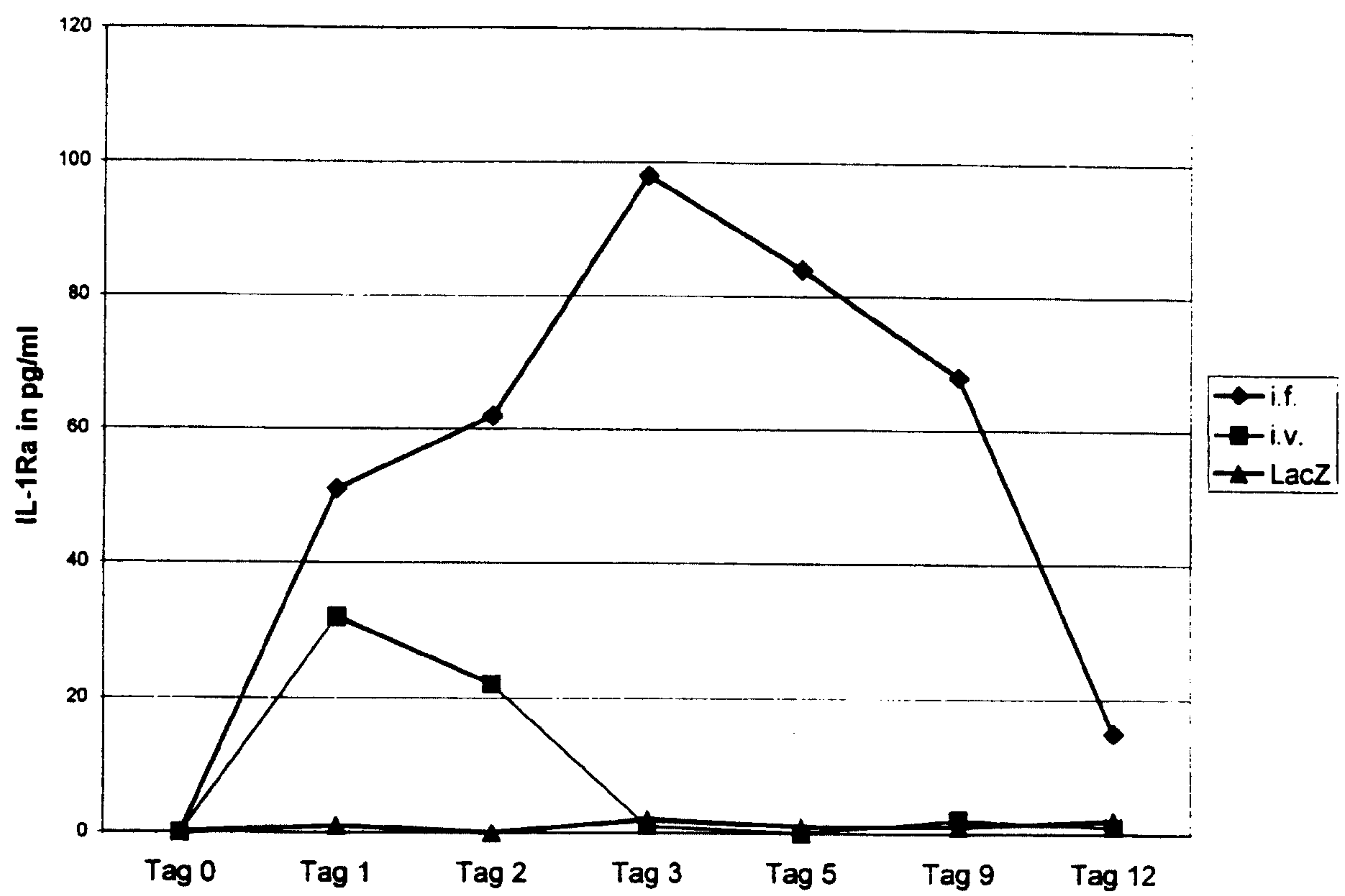


Fig. 5



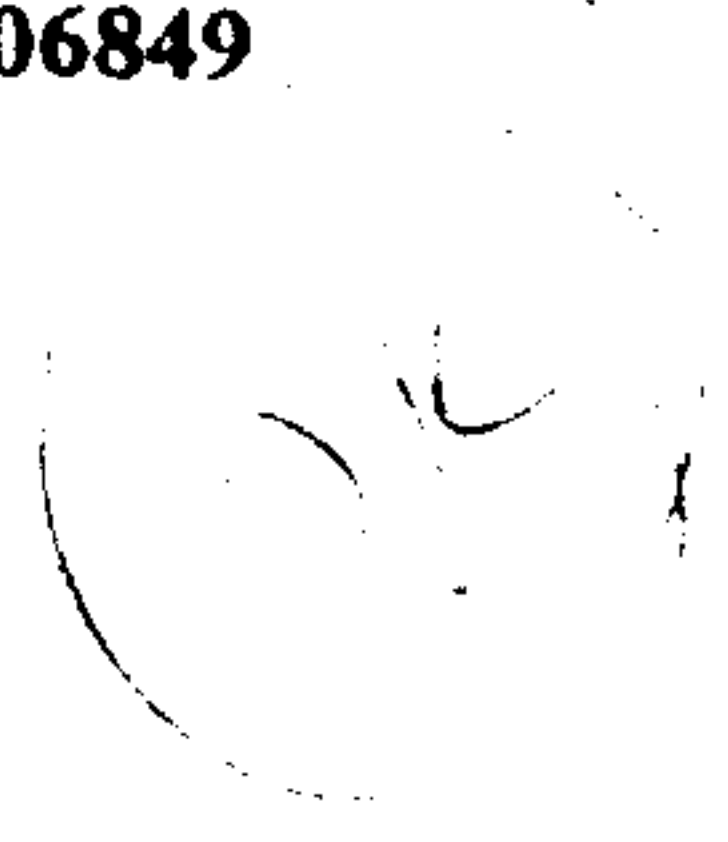
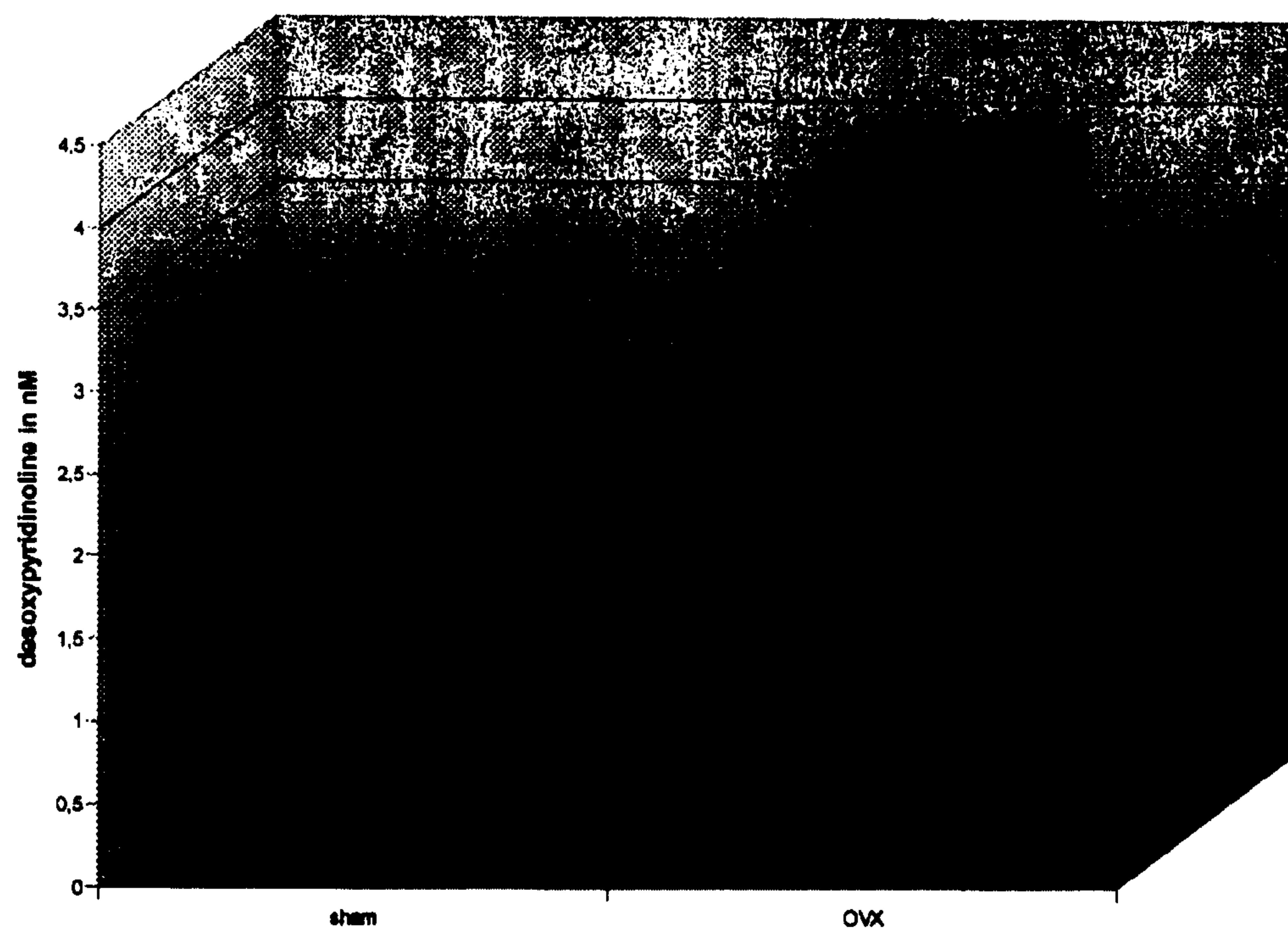
**Fig. 6**

Fig. 7

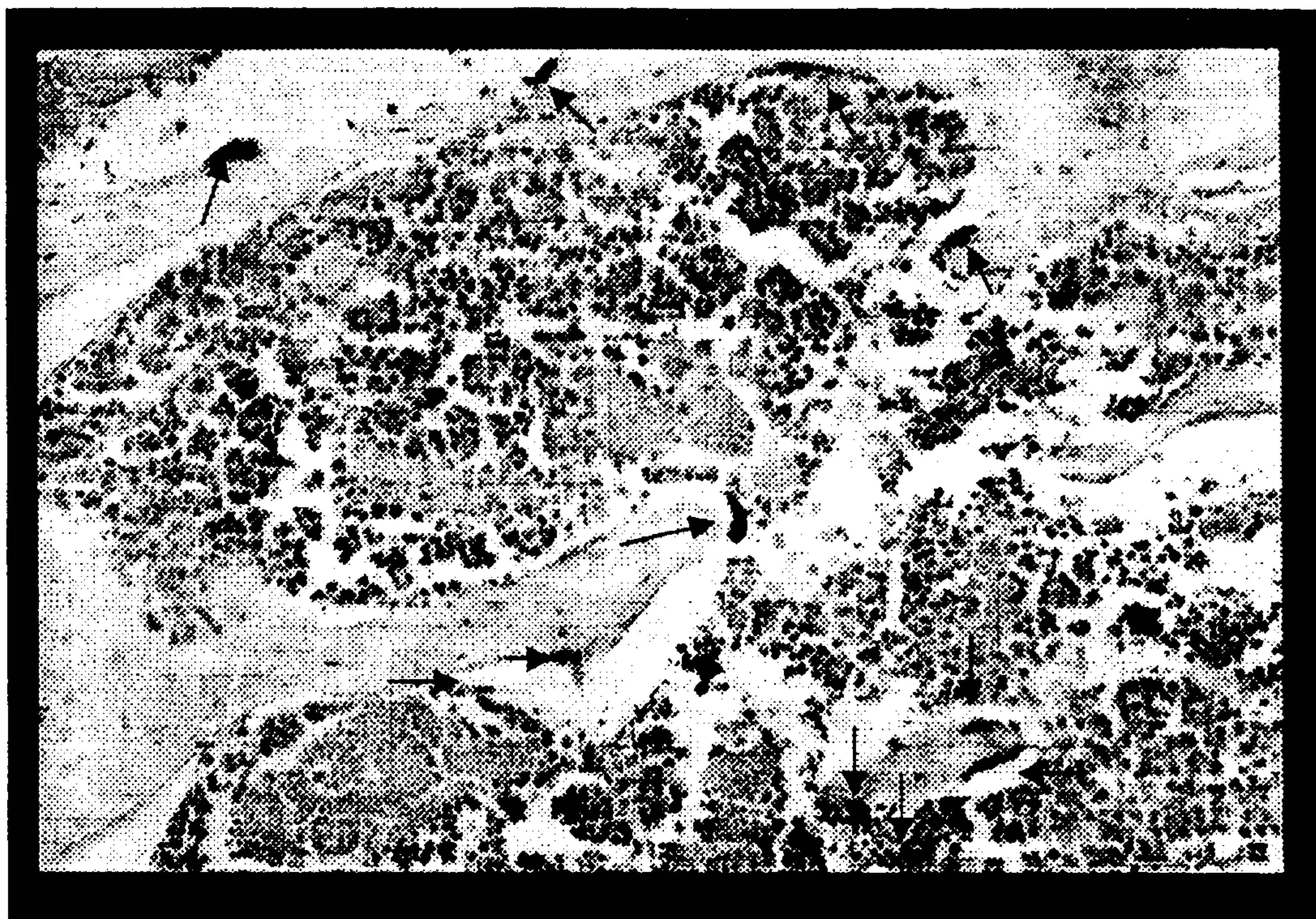


Fig. 8

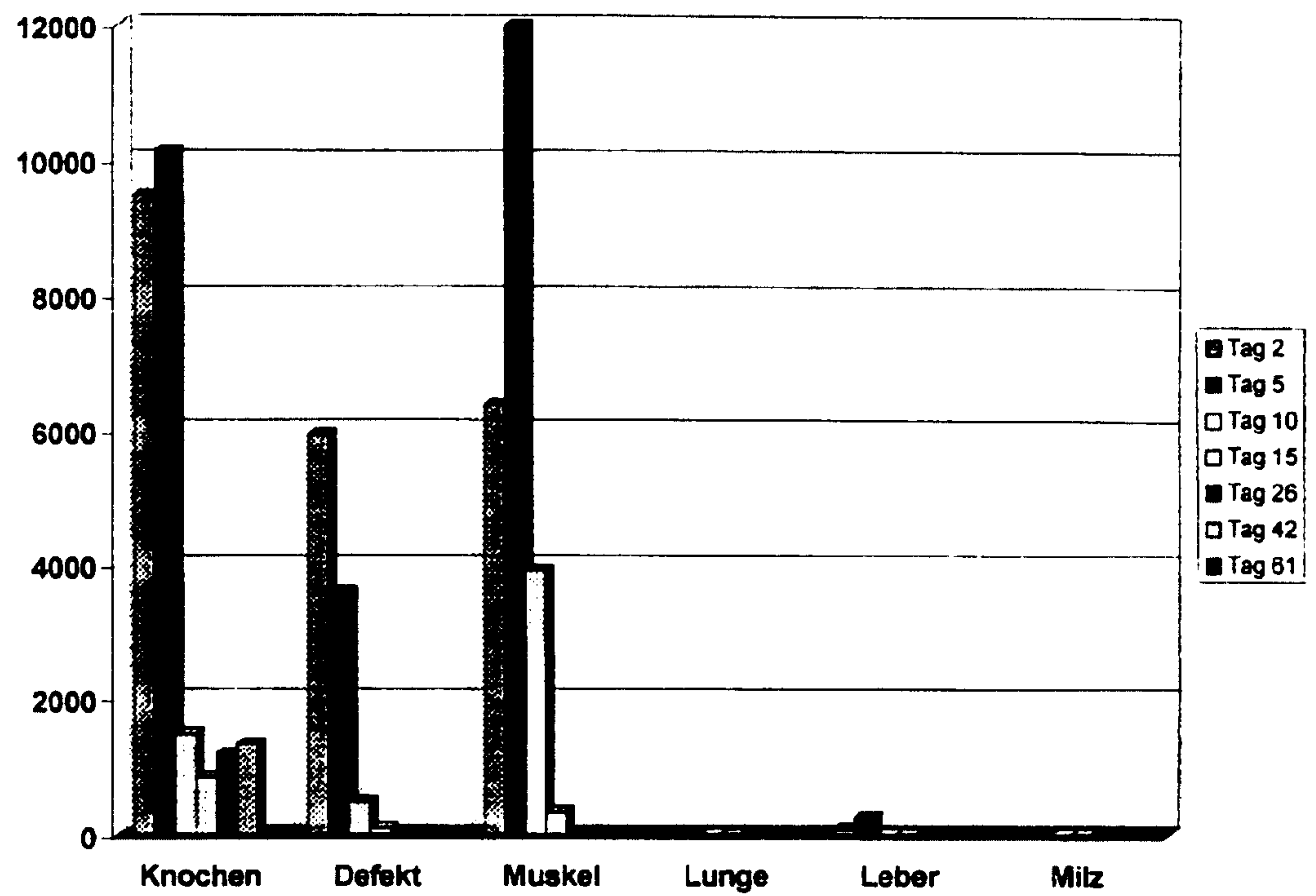


Fig. 9



Fig. 10

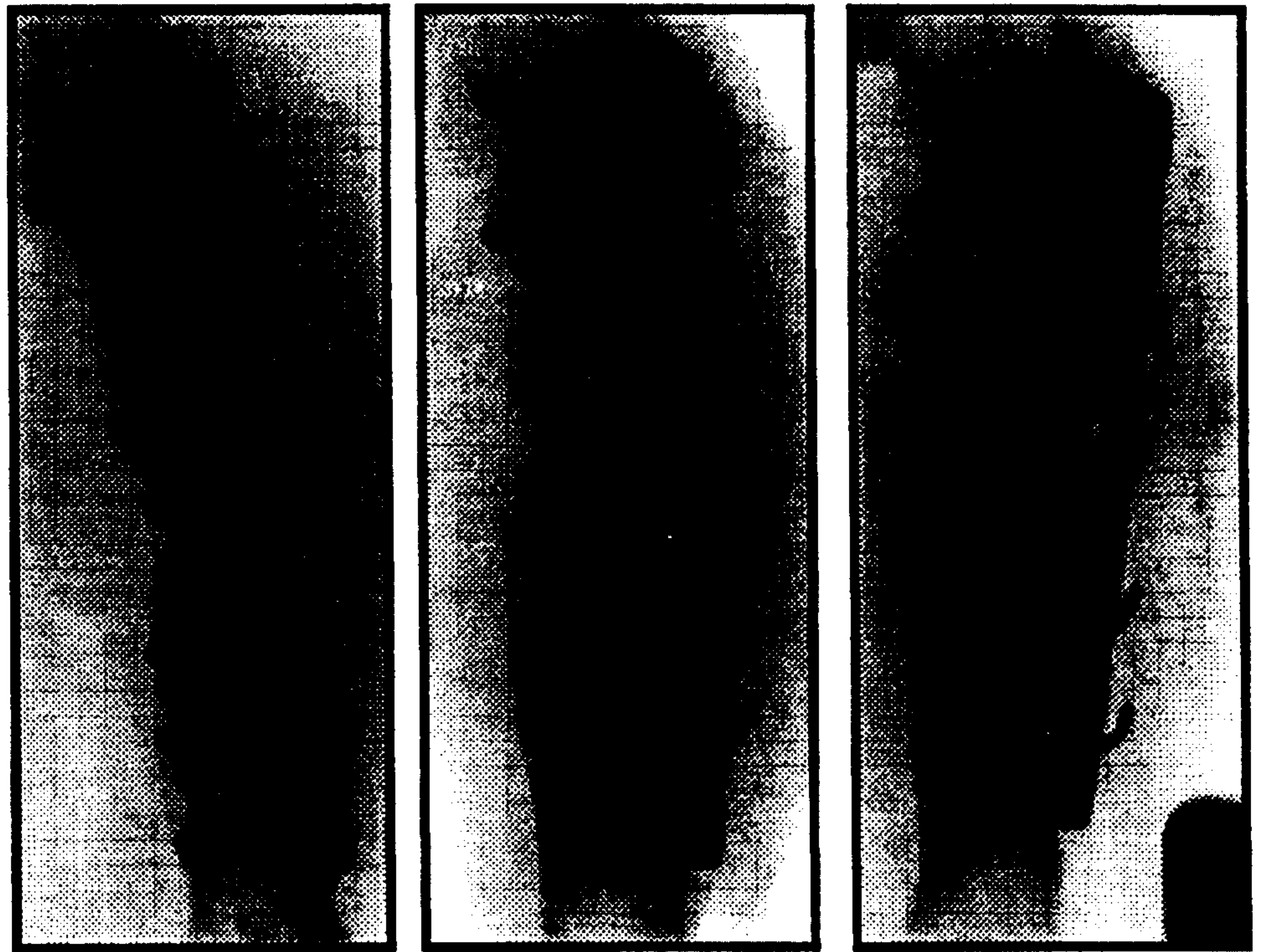


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Fig. 11

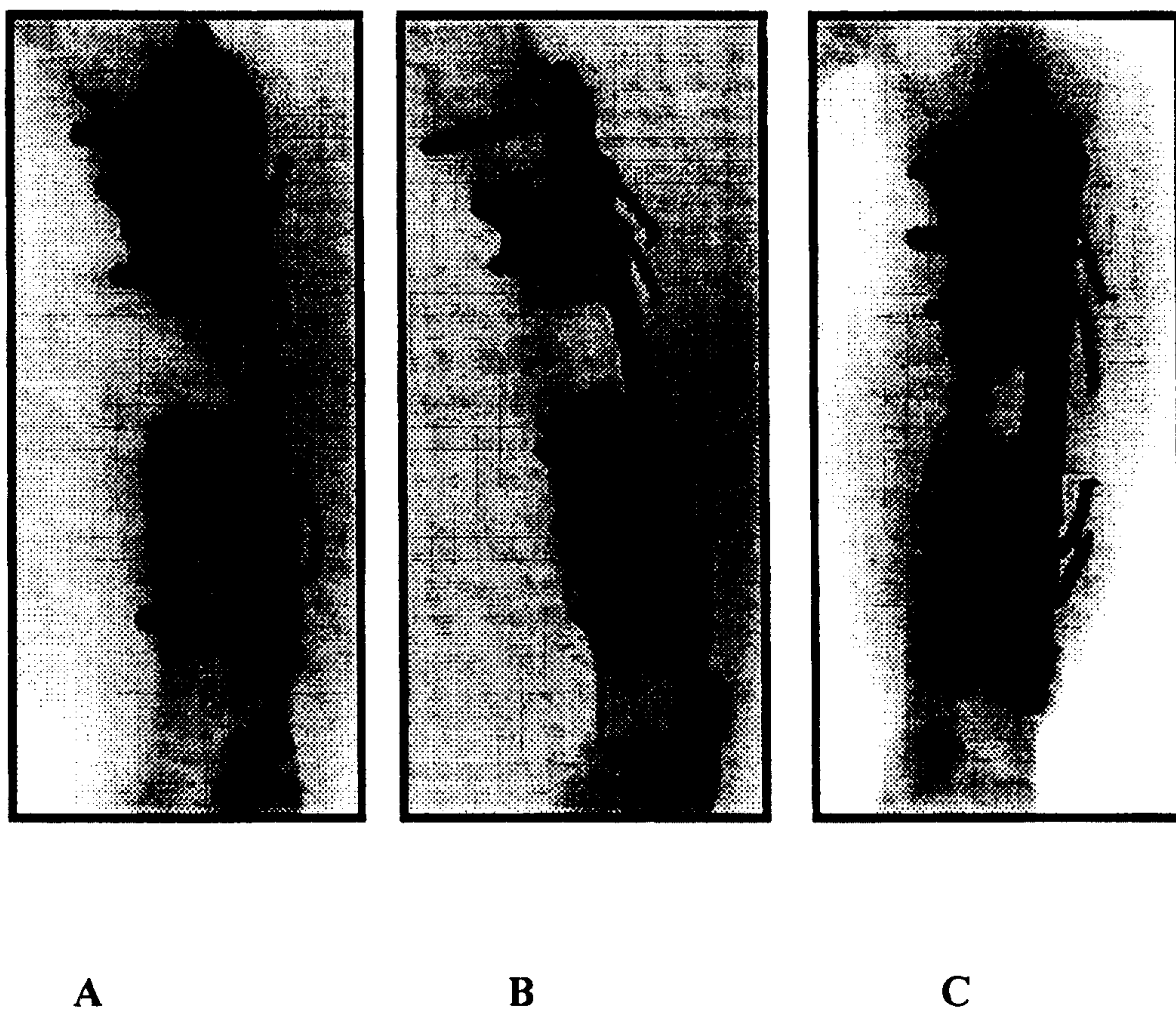


A

B

C

Fig. 12



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Fig. 13

