JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **10** (1999) 653–658

Biodegradable drug delivery system for the treatment of bone infection and repair

L. DI SILVIO
IRC in Biomedical Materials, Institute of Orthopaedics (UCL), Brockley Hill, Stanmore, Middlesex HA7 4LP, UK

W. BONFIELD
IRC in Biomedical Materials, Queen Mary and Westfield College, Mile End Road, London E1 4NS, UK

A drug delivery system (DDS) which provides a sustained release of antibiotics at the focal site either singly, or in combination with a bone stimulating factor could both eliminate infection and increase the number of potentially healthy osteogenic cells. In this study, we address the use of a degradable gelatin DDS, for the combined release of therapeutic levels of both gentamicin and growth hormone (GH). An initial bolus release was observed during the first 24 h followed by a reduced, but sustained, release for both drugs up to 14 days. Bioactivity of gentamicin was demonstrated by growth inhibition of *Staphylococcus aureus* for over 96 h with a mean zone of inhibition of 29.4 mm ($\pm$ 0.19) for the time period studied. Furthermore, GH was shown to have a direct effect on primary human osteoblast-like (HOB) cells, stimulating proliferation and enhancing their differentiation. Site-specific drug delivery offers the advantage of localizing a drug directly at the target site, thus minimizing systemic effects. The results of this study suggest that gelatin is a good DDS for the combined release of drugs. In addition, gelatin is both biocompatible and biodegradable, thus making it a promising DDS for the management of acute and chronic bone and tissue infection such as osteomyelitis.

1. Introduction

Novel biomaterials which provide effective relief from a range of painful, crippling and sometimes life-threatening disorders have been, and continue to be developed. In the wake of this progress, however, has come the problem of device-associated infection [1–3]. The rate of infection in major surgery, even with prophylactic antibiotics is 0.5–6% [4, 5]. In trauma cases, where there is contamination, vascular injury and exposed bone, the infection rate can rise from 2.5–12% and higher [6, 7]. In addition to the detrimental effect of these complications on the actual patient, the cost of medical care of patients with severe infection is very high [1].

The fate of the biomaterial surface is dependent upon several factors including adsorption of matrix proteins. When a biomaterial surface is exposed to a biological environment it rapidly acquires a layer of glycoproteins, such as albumin, collagen and fibronectin. Biomaterial implants provide a suitable adhesive substratum for bacterial colonization. Bacteria have the ability to rapidly colonize the surface of biomaterials, thus preventing the attachment of tissue cells. Tissue cells compete with bacterial cells for colonization of the biomaterial surface, thus reducing host response and tissue integration whilst increasing the risk of infection. A bone infection can spread very rapidly and can involve the medullary space, cortex and periosteum. In certain cases the infection is capable of inducing chronic inflammation, tissue necrosis or even life-threatening conditions such as septicaemia. Once the organisms reach the bone marrow there can be extensive suppuration through the bone marrow which can cause subperiostial abscesses, which can lead to further soft tissue infection [8].

Osteomyelitis is a clinical condition that is difficult to treat and thus much effort goes into avoiding its occurrence both pre- and post-operatively. The major problems are associated with poor antibiotic distribution at the site of infection due to limited blood circulation to the surrounding skeletal tissue [9].

The resistance of devices to bacterial colonization can be increased by modifying the biomaterial surface. Polyurethanes, for example, have been produced with increased hydrophilic surfaces which appear to reduce the growth of *Staphylococcus epidermidis*. Hydrophilicity of surfaces can be increased by irradiation techniques which introduce functional groups at the surfaces of polymeric materials [3]. Another approach has been to incorporate antimicrobial agents such as flucloxacillin into polyurethane and iodine into polyvinyl fluoride [10]. Elliot *et al*. [11] described a method whereby low amperage electric currents were used as a means of protecting biomaterials against colonization.

Whilst these developments provide possible methods to control material-associated and trauma infections, it is clear that microbes are highly adaptive organisms, and hence the search for a more definitive control mechanism continues.

Under normal circumstances the host defences are able to deal with the infection, but in cases of severe trauma rapid bacterial colonization can occur [2, 8]. As a result, the repair process may be delayed due to poor cell number and reduced cellular activity. In these cases, the release of a bone-stimulating factor in combination with an antibiotic can have the further advantage of stimulating osteogenic cells in the vicinity to proliferate and differentiate [12, 13].

Controlled drug delivery has caused considerable interest in recent years, with particular attention being centered on the use of biodegradable systems. A problem common to most systems is that they display either a constant release rate or the release is dependent upon degradation of the system with time. Numerous methods for drug delivery have been described in the literature and include encapsulation, membrane-enclosed reservoir devices, and monolithic systems [14–16].

Our approach was to develop a monolithic microspherical drug delivery system (DDS) where the drugs are dispersed within a biodegradable, biocompatible matrix. Gelatin, a naturally occurring non-toxic polymer was the matrix used. The use of gelatin as a DDS for peptides has been described previously [17, 18]. In this study we investigated its potential to release both gentamicin and growth hormone (GH) in a controlled manner. The bioactivity of the gentamicin released was tested against *S. aureus* and the effect of GH was evaluated using human osteoblast-like (HOB) cells.

2. Materials and methods

2.1. Preparation of microspheres

The microspheres were prepared using a method previously described [18]. Briefly, 20 g of 300 Bloom gelatin (Swine skin Type A, Sigma) was dissolved in 100 ml of sterile water. The solution was then divided into two aliquots, one of which was left plain as a control. The drugs gentamicin (Roussel, gentamicin base as sulfate 80 mg) and human GH (Serono 8IU) were added directly to the second aliquot of the gelatin solution and care was taken to ensure an even dispersion. Microsphere size was controlled by using a fine gage 23G needle and they were formed by forcing the gelatin mixture through a pre-heated syringe into chilled paraffin oil where they solidified and collected at the bottom. The paraffin oil was removed and the microspheres were collected and washed three times in chloroform to remove all traces of the oil. Cross-linking of the microspheres was achieved by placing them in a vapor of 25% glutaraldehyde for 48 h, thus minimizing any toxic effect. Successful cross-linking was indicated by a change from straw color to a deep yellow color. They were washed several times in phosphate-buffered saline (PBS) and dried in a stream of cool air overnight, to allow the evaporation of any chloroform that may have still been present and also any glutaraldehyde that may have got onto the microspheres.

2.2. Elution of gentamicin and GH

The elution of the drugs was monitored in PBS by adding 2.5 ml of solution to each 0.1 g of microspheres (mean = 28 microspheres). The release was carried out at 37 °C on a continuous rolling mixer. The PBS was removed and replaced with 2.5 ml of fresh solution, after 1 h and then daily; the elution fluid was assayed for gentamicin and GH.

2.3. Cytotoxicity testing

Plain and GH-loaded gelatin microspheres (0.1g) were placed in 2.5 ml of PBS and incubated at 37 °C on a rotating mixer for a period of 24 h. Eluates were removed and used to test the direct effect of any toxic degradation products on the cells that would be exposed to them. The Neutral Red uptake (NR) cytotoxicity assay which measures cell survival, based on the ability of viable cells to accumulate NR in lysosomes after the dye has diffused through the cell membrane, was used. Any changes in the cell surface or the lysosomal membrane lead to changes resulting in decreased uptake and accumulation of the dye.

A primary HOB cell model was used for the cytocompatibility testing. These cells were obtained by a method previously described [19]. HOB cells were seeded at a density of 6000 cells/well in complete Dulbecco's minimal essential medium (DMEM) in a 96-well plate. These were allowed to grow for a period of 24 h, which usually resulted in approximately 60% confluence, after which the medium was replaced with elution fluids from both plain and growth hormone loaded microspheres (100 µl/well). The plate was incubated for a further 24 h at 37 °C, and then the elution fluids were replaced with the dye solution (50 $\mu g\,ml^{-1}$) for a further 3 h to allow dye uptake by the cells. The cells were washed with 0.5% formaldehyde/1% calcium chloride solution and the dye extracted using a solution of 1% acetic acid/50% ethanol. They were measured at 540 nm on a Dynatech plate reader.

2.4. Growth hormone assay

The GH was measured using an ''in-house'' enzyme-linked immunoadsorbent assay (ELISA). The coating antibody was a polyclonal guinea-pig IgG fraction and the conjugated second antibody a peroxidase-labeled Fab′-fragment of guinea-pig antihuman GH. 3,3′–5,5′-tetramethylbenzidine (TMB) was the substrate for the enzymatic reaction. Optical density was measured at 490 nm with a reference wavelength of 650 nm (Dynatech 800 plate reader). The standard used was 22K-recombinant human GH (antibodies and standard GH were generous gifts from Novo Nordisk A/S, Gentofte, Denmark).

2.5. Gentamicin assay

Gentamicin was assayed using an enzyme immunoassay EMIT (Boehring, UK). Eluates were assayed neat and in dilution and values determined from the standard curve. Eluates from plain gelatin microspheres were tested to ensure that there was no matrix effect and quality

controls were measured at regular intervals to check for drift between the measured samples.

2.6. Assessment of biological activity of gentamicin

S. aureus was used to set up the initial culture in Bacto heart infusion broth (Difco). Plates for the inhibition zone experiment were prepared using a 3.8% heart agar (Oxoid) and a 0.8% high-binding agar gel (Sigma) solution. The solution was autoclaved and allowed to cool to 50 °C prior to the addition of 25 ml of bacterial culture; this was shaken and returned to the waterbath. The heart agar solution (25 ml) was added to each petri dish using aseptic techniques and allowed to set at room temperature. Using a sterile hole punch (approximately 0.5 cm in diameter) five holes were punched in the gel; one gelatin microsphere was placed in each hole which was also filled with liquid heart medium. The plates were then placed in a warm room at 37 °C for 24 h. Following this incubation time, the plates were checked for any clear zones around the microspheres and the distance was measured.

The minimum inhibitory concentration (MIC) of gentamicin for *S. aureus* culture was determined. For the kill curve 0.04 g of gentamicin containing microspheres, were placed in 75 ml of liquid culture of *S. aureus*. At 0, 24, 48 and 72 h, 2 ml were removed from each and serially diluted and plated out on to agar plates. Individual colonies were counted and expressed as colony-forming units (CFU ml^{-1}).

2.7. Tritiated thymidine labeling [^{3}H]-TdR of cells in culture

Primary HOB cells were obtained from trabecular bone fragments from femoral heads of patients undergoing surgery for total joint replacement. The cells were fully characterized prior to use [19]. [^{3}H]-TdR incorporation into the DNA of HOB cells was assessed as follows; cells were incubated in 24-well plates at a density of 30 000 cells per well in DMEM supplemented with 10% fetal calf serum until they were 70–80% confluent. The cells were then cultured in serum-free DMEM in order to arrest cell growth for 16 h prior to the addition of fresh control or test medium. The test medium contained 50 ng ml^{-1} of human GH, which was added to the arrested cells with 1 μCi ml^{-1} of [^{3}H]-TdR (Amersham, International plc, UK) and incubated for 24 h. Following this, the cells were digested using a papain digest solution, containing 1 μl papain suspension (type III, Sigma, Poole, UK) in 1 ml PBS supplemented with 5 mM cysteine hydrochloride (Merck, UK) and 5 mM ethylenediamine tetra-acetic acid (EDTA, Merck, UK) at pH 5.7 for 24 h at 60 °C. One hundred microliters of the digest was transferred to a scintillation vial and the amount of radiolabel incorporated was measured on a scintillation counter.

2.8. Measurement of total DNA

The DNA content of the cells was measured using a modification of the method of Kapuscinski and Skoczylas [20]. A 20 μl aliquot of papain digested cells was added to 480 μl 10 mM NaCl, followed by 300 μl distilled water, 100 μl Tris (40 mM, pH 7.4) and 100 ml of a 2 mM 4′,6-diaminodino-2-phenylindole.2HCl (DAPI; Sigma) solution. After thorough mixing the samples were read in a LS2B fluorimeter (Perkin-Elmer, UK) at a wavelength of 460 nm. The standards contained 12.5, 25, 50, 250 ng ml^{-1} DNA (Calf thymus, Type 1 from Sigma, UK).

2.9. Biochemical measurement of alkaline phosphatase (ALP)

For the biochemical analysis cells were plated at a density of 60,000 cells per well and cultured in medium containing 50 ng ml of GH for a period of 21 days. ALP activity in the cell lysate and the medium was determined using a COBAS-BIO (Roche, UK) centrifugal analyzer. The assay measures the release of *p*-nitrophenol from *p*-nitrophenol phosphate (PNPP) at 37 °C in buffer containing 1 M diethanolamine (DEA), 10 mM PNPP and 0.5 mM $MgCl_2$ and 0.22 M NaCl, pH 9.8 (Merck, UK).

Statistical analysis of the data presented was performed using the Student's *t*-test (paired) to determine whether any significant differences existed between the test groups and the control.

3. Results

Both gentamicin and GH are soluble and a rapid release was observed during the first 24 h for both. Gentamicin was released in two phases (Fig. 1). An initial high dose was released, with approximately 79% of the total amount incorporated being released during the first 24 h by normal Fick diffusion mechanism (Fig. 2) [18]. This was followed by a second phase of sustained release up to 14 days. A similar release pattern was observed for the GH with the greatest release during the first 24 h.

A significant reduction in cell viability was seen in

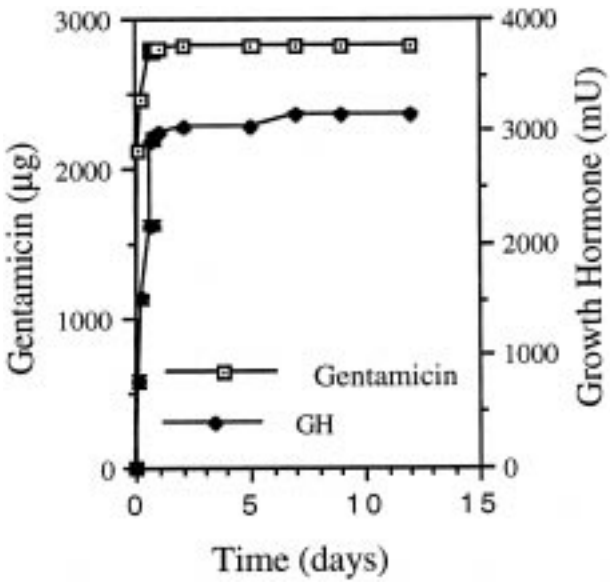

Figure 1 The combined release of gentamicin and GH from gelatin microspheres over a 14-day period.

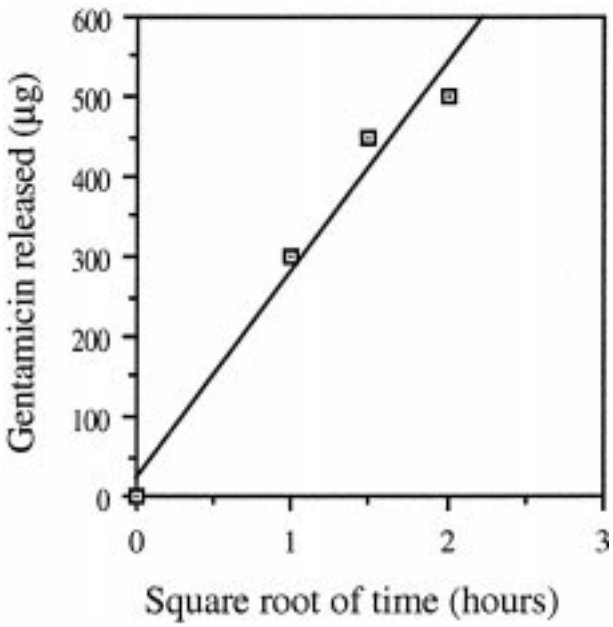

Figure 2 Mass of gentamicin released during the first 2 h. A straight line through the origin was observed during the initial release phase, thus suggesting diffusion controlled release.

those cells cultured in the plain gelatin elution fluid (Fig. 3) ($P < 0.05$). The reduction observed was probably a consequence of nutrient deprivation of the cells and not an indication of cytotoxicity. A slight reduction in cell viability was observed in the GH containing elution fluid but this was not significant. The increase in cell viability in the GH-loaded gelatin may have been a direct effect on the cells by the GH released into the medium.

Growth inhibition of *S. aureus* demonstrated that the gentamicin released was active for over 96 h, and the mean zone of inhibition was 29.4 ($\pm$ 0.19) mm for the time period studied (Fig. 4).

During the first 24 h no significant difference was observed between the controls and the treated microspheres. A rapid drop in bacterial colonies was observed

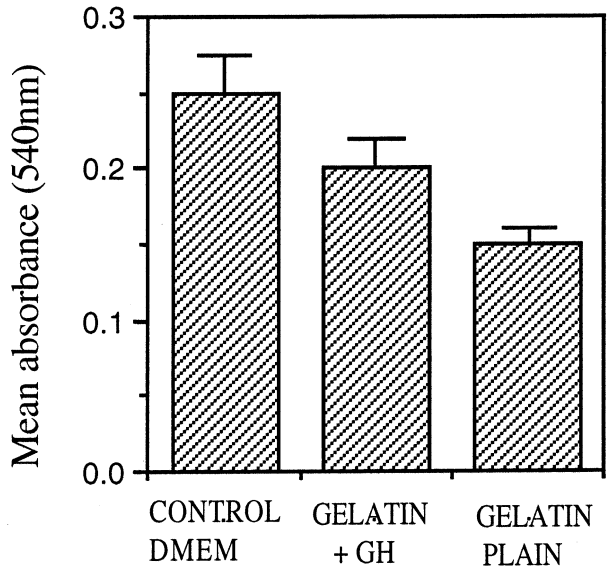

Figure 3 NRU assay for gelatin elution fluids (Mean + SD). HOB cells were incubated in control medium (DMEM, 10% FCS), elution fluid from GH-loaded microspheres and from plain gelatin.

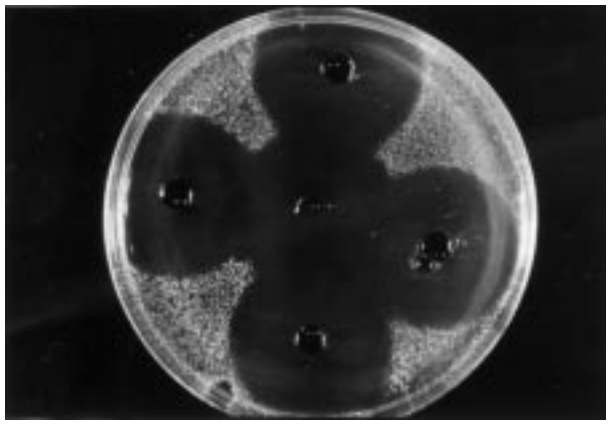

Figure 4 Gentamicin release from the microspheres on a *S. aureus*-containing plate showing inhibition zones.

at 48 h and by 72 h the bacterial count was down to zero in those cultures in the presence of the gentamicin-loaded microspheres for all batches tested (Fig. 5). In the controls, the bacterial count (10^8 CFU ml^{-1}) remained constant throughout the test period.

GH stimulated HOB cell proliferation in a dose-dependent manner with a significant increase in ^{3}H-thymidine incorporation (Fig. 6). Maximal stimulation occurred at 25 ng ml^{-1} of GH. ALP is a marker of HOB cell differentiation and in the presence of GH a significant increase in activity was observed ($P < 0.05$) from day 7 onwards. A peak in ALP activity was observed in both the control and GH-stimulated HOB cells at 14 days (Fig. 7).

4. Discussion

When a foreign body such as a biomaterial is implanted in the body, a wound healing process takes place following trauma induced by the implantation. The process includes an inflammatory phase and a remodeling phase. The acute inflammatory phase is usually

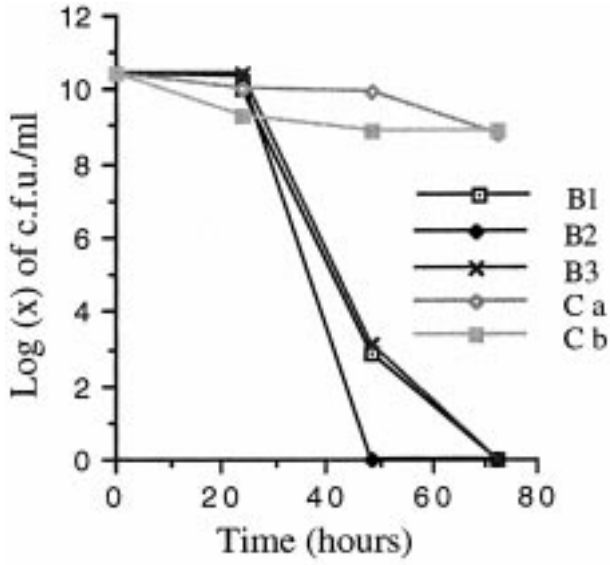

Figure 5 Kill curve for bacterial colonies of *S. aureus* in the presence of control plain gelatin microspheres and microspheres containing gentamicin. Three batches of test (B1,2,3) and two batches of control (Ca,b) microspheres were tested.

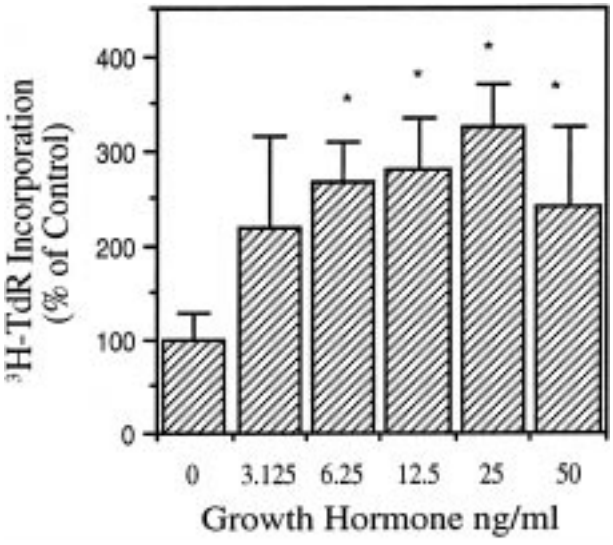

Figure 6 The incorporation of ^{3}H-thymidine in DNA of HOB cells stimulated with increasing doses of GH. The results represent the mean expressed as a percentage of the control (+ SD) ($^*P < 0.05$).

controlled by the host defence mechanism and involves many cells including neutrophils, monocytes and macrophages. If the inflammatory response is persistent, then chronic inflammation results, involving multinucleated giant cells [21]. In these cases, a DDS which released pharmacologically active agents at the target site, in a controlled manner would be desirable.

In this study, we have achieved a drug delivery system for the combined release of gentamicin and GH, which has an initial bolus release followed by a sustained release for a period of 14 days. Gelatin serves as an ideal colloid carrier for the controlled release of drugs [22]. It offers numerous advantages over existing systems: it is a natural polymer, which is biodegradable, non-toxic and biocompatible. Furthermore, its preparation does not involve a thermal reaction, or the addition of a crosslinking agent, thus assuring physical and chemical stability of the incorporated drugs.

The release of agents from monolithic systems usually display zero-order kinetics, with release rates diminishing with time [14–16]. The release of gentamicin and GH in this study was biphasic. During the first phase a large amount of drug was released within the first 24 h. The release kinetics for the gelatin microspheres have previously been described [18]. An initial fast release, or burst effect, was observed followed by a much slower, first-order release. Several factors may influence the release kinetics of drugs from matrices; Forni *et al.* [23] suggested that drug loading may influence the dynamic swelling of crosslinked gelatin and subsequent release of the drug. Both gentamicin and GH are highly soluble; however, only 25% of the total GH incorporated was released compared to 79% of gentamicin. The difference observed in the amount of drug released was probably due to the difference in size of the two molecules, gentamicin being much smaller (MW 300) than GH (MW 22 000).

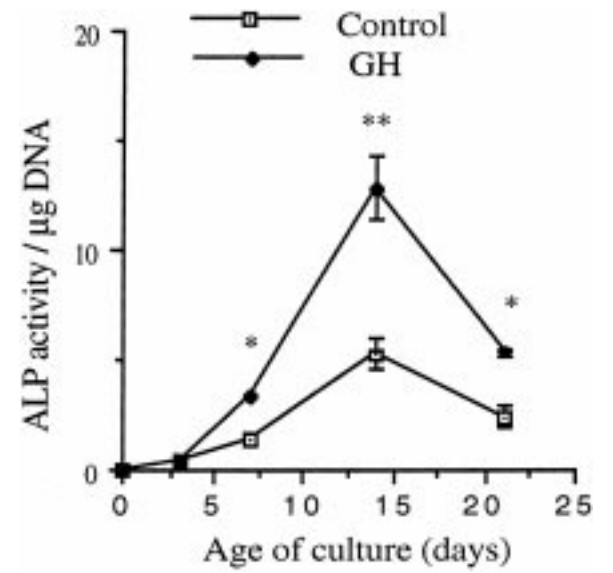

Figure 7 ALP activity expressed per DNA for control and GH stimulated cells cultured for a period of 21 days.

Bone cells produce and secrete numerous growth factors; these have been shown to influence cell proliferation and differentiation [24, 25]. GH significantly increased proliferation and ALP activity of the HOB cells. GH has been shown to have a direct effect on bone cells [26]. By increasing bioavailability of growth factors in the bone cell microenvironment, it may be possible to regulate and mediate bone cell metabolism. Further studies, however, are necessary to establish the exact role of GH and its mechanism of action.

The microspheres in this study were shown to be bactericidal; the results suggest that after a high dose at 24 h the dose released was of equal potency up to 96 h and this was further confirmed by the kill curve results. From the results it would appear that the microspheres can release therapeutic levels of antibiotics. However, levels achieved *in vitro* and *in vivo* can differ greatly, and data on the pharmacokinetics of gentamicin release at the target site would need to be determined.

5. Conclusion

An increase in local bioavailability of drugs could be of potential use in trauma cases where infection may reduce the healing time. The combined release of gentamicin and GH could have a significant effect on cells in the vicinity, by enhancing both the proliferation and the differentiation of cells. However, further work is required to assess the *in vivo* effect on the healing capabilities of the released drugs and the effect of the degradation processes.

Acknowledgment

The continued support of the EPSRC for the IRC core program is gratefully acknowledged together with the assistance of Mr P. Herbert for the microbiology.

References

1. D. J. STICKLER and R. J. C. MCLEAN, *Cells Mater.* **5** (1995) 167.
2. G. D. CHRISTENSEN, L. M. BADDOUR, D. L. HASTY, J. E. I. LOWRANCE and W. A. SIMPSON, in ''Infections associated with indwelling medical devices'', edited by A. L. Bisno and F. A. Waldvogel (American Society for Microbiology, 1989) p. 27.

3. A. G. GRISTINA, G. GIRIDHAR, B. L. GABRIEL, P. T. NAYLOR and Q. N. MYRVIK, *Int. J. Artif. Organs* **16** (1993) 755.
4. E. MULLER, S. TAKEDA, D. GOLDMAN and G. PIER, *Infect. Immun.* **59** (1991) 3323.
5. S. H. DOUGHERTY and R. L. SIMMONS, *Curr. Probl. Surg.* **19** (1982) 221.
6. *Idem., ibid.* **19** (1982) 265.
7. A. G. GRISTINA, L. X. WEBB and E. BARTH, in ''Infections in the orthopaedic patient'' edited by R. Coombs and R. Fitzgerald (Butterworths, London, 1989) p. 30.
8. A. G. GRISTINA, *Clin. Orthop. Rel. Res.* **298** (1994) 106.
9. R. H. FITZGERALD JR, *Orthop. Clin. N. Amer.* **15** (1984) 537.
10. B. JANSEN and G. PETERS, *J. Hosp. Infect.* **19** (1991) 83.
11. T. S. J. ELIOTT, J. HOLFORD, P. SISSON and P. BRYNE, *J. Med. Microbiol.* **33** (1990) 2.
12. V. V. VILJANEN, T. J. GAO, T. C. LINDHOLM, T. S. LINDHOLM and B. KOMMONEN, *Int. J. Oral Maxillofac. Surg.* **25** (1996) 217.
13. G, MOREL, B. CHAVASSIEUX, B. BARENTON, P. M. DUBOIS, P. J. MEUNIER and B. BOIVIN, *Cell Tissue Res.* **273** (1993) 279.
14. J. WRIGHT, S. K. CHANDRASEKARAN, R. GALE and D. A. SWANSON, *Controlled Release Systems*, AIChE Symposium Series **77** (1981) p. 62.
15. L. M. SANDERS, G. I. MCRAE, K. M. VITALE and B. A. KELL, *J. Controlled Rel.* **2** (1985) 187.
16. J. R. CARDINAL, KIM SUNG WAN, SONG SUK-ZU, LEE EUN SOO and C. KIM SUNG-HO, *Controlled Release Systems,* AIChE Symposium Series **77** (1981) 52.
17. L. DI SILVIO, R. G. COURTENEY-HARRIS and S. DOWNES, *J. Mater. Sci.: Mater. Med.* **5** (1994) 819.
18. L. DI SILVIO, N. GURAV, M. V. KAYSER, M. BRADEN and S. DOWNES, *Biomaterials* **15** (1994) 931.
19. L. DI SILVIO, PhD Thesis, University of London, 1995.
20. J. KAPUSCINSKI and B. SKOCZYLAS, *Anal. Biochem.* **83** (1977) 252.
21. J. M. ANDERSON and K. M. MILLER, *Biomaterials* **5** (1984) 10.
22. R. NARAYANI and K. PANDURANGA ROA, *J. Microencapsulation* **11** (1994) 69.
23. F. FORNI, M. A. VANDELLI and R. CAMERIN, *ibid.* **9** (1992) 29.
24. E. CANALIS, T. MCCARTHY and M. CENTRELLA, *J. Clin. Invest.* **81** (1988) 277.
25. C. C. KASPERK, J. E. WERGEDAL, S. MOHAN, D. L. LONG, K. H. W. LAU and D. J. BAYLINK, *Growth Factors* **3** (1990) 147.
26. M. KASSEM, W. BLUM, J. RISTELLI, L. MOSEKILDE and E. F., *Calcif. Tissue Int.* **52** (1993) 222.

Received 1 December 1998
and accepted 18 February 1999