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A comparison between two methacrylate cements as delivery systems for bioactive human growth hormone

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Two methacrylate orthopaedic bone cements were compared as delivery systems for bioactive human growth hormone (hGH). These were London Hospital Bone Cement (LHBC) and a poly(methylmethacrylate) (PMMA) bone cement. A uniquely precise bioassay for hGH, named ESTA, was used. It was adapted to assess both the bioactivity of the hormone released from hGH loaded cements and also the *in vitro* cytotoxicity of the cements themselves. Eluates from both cements proved cytotoxic, this being most pronounced for LHBC. The cytotoxicity could be readily diluted out, and a lengthy exposure time (> 24 h) was required to kill the cells. Both cements released similar quantities of bioactive hGH, which were ~0.6% of that originally incorporated. This could potentially provide significantly high local concentrations of the hormone to the tissues surrounding the implant. Approximately double quantities of hormone were released as measured by immunoassay, leading to significant decreases ($p = < 0.0001$) in the B:I ratios of the released hormone. The effect was greatest for LHBC. Our results suggest that the structural integrity of the hGH molecules released from LHBC were the most compromised.

1. Introduction

Poly(methylmethacrylate) (PMMA) bone cement has been extensively used as a fixative for total hip replacements [1]. Although it has remained the material of choice, it has several disadvantages [2]. These include excessive heat of polymerization, when temperatures of 80–90 °C are achieved, shrinkage of the cement during polymerization, biomechanical deficiencies and most relevant of all, necrotic effects on the surrounding tissues.

To overcome these problems a cement was formulated called London Hospital Bone Cement (LHBC) which was based upon a clinically successful dental material [3, 4]. LHBC is based upon poly(ethylmethacrylate) powder (PEMA) as opposed to PMMA, with n-butylmethacrylate as the monomer. PMMA cement utilizes methylmethacrylate as the monomer. LHBC exhibits some favourable biomechanical properties when compared to PMMA [5], and has several additional advantages over PMMA as reviewed by Patel and Braden [6].

The toxic effects of both of these cements on surrounding tissues are thought to be largely due to the release of free monomer from the final preparations, with lower monomer release from freshly mixed LHBC [7]. One study showed that the monomer n-butyl methacrylate was slightly more toxic than the monomer methylmethacrylate, when injected intraperitoneally into mice [8]. However, Mir [9] reported that n-butyl methacrylate was the less toxic monomer when tested upon isolated perfused rabbit hearts.

The biological reaction to LHBC *en mass* was investigated by Revell *et al.* [10]. He observed a macrophage response to implanted beads of both PEMA and PMMA polymer powders, when these were inserted subcutaneously into rats. However, implantation of the corresponding cements into the paraspinal musculature of rats, resulted in less tissue damage adjacent to LHBC than PMMA, with a reduced macrophage and giant cell reaction.

Recombinant human growth hormone (hGH) has been incorporated into PMMA, and its *in vitro* release was demonstrated, together with an enhancement of early osteoid formation in rabbits in *in vivo* studies [11]. A subsequent clinical trial of hGH-loaded PMMA showed elevated local levels of both hGH and insulin-like growth factor I, following implantation [12].

The aim of the present study was to compare the release of hGH from PMMA with that from LHBC. This hormone is a complex protein molecule with a molecular weight of 22 kDa, and a high degree of structural integrity is required for its biological

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efficacy. Because of the potential damage to this structure when hGH is incorporated into an organic matrix and subjected to the high temperatures of polymerization, it is preferable to investigate the bio-, as opposed to the immunoactivity, of the hormone. In previous studies the release of hGH from PMMA was monitored using an immunoassay [11, 13]. We now have available a bioassay, in the form of ESTA bioassay system, which allows for the first time, measurement of the bioactivity of the released hGH in a highly quantitative manner.

The ESTA bioassay for the measurement of hGH [14, 15] uses the growth hormone responsive rat lymphoma Nb2 cell line as the target cell [16]. In this colorimetric bioassay the response of the cells to the hormone is detected by the addition of the yellow tetrazolium salt MTT. This is bioreduced by the activated Nb2 cells to generate a purple formazan product.

2. Materials and methods

2.1. Media and substances

PMMA in the form of CMW-3 was obtained from Dentsply, Exeter UK. LHBC was obtained from Bonar Cole Polymers Ltd, Newton Aycliffe, Co Durham, UK. The radio opaque agent for both cements is barium sulphate. In LHBC it is contained within the polymer beads, whereas for PMMA it resides within the interbead matrix. For both cements benzoyl peroxide in the powder, and N,N dimethyl-p-toluidine in the monomer initiate polymerization. Recombinant hGH (Austrophin) was a kind gift from CSL Ltd, Victoria, Australia. RPMI 1640 medium, L-glutamine, penicillin/streptomycin and tissue culture ware (Nunc) were obtained from Gibco BRL (Life Technologies), Paisley, UK. Horse and fetal calf serum were purchased from Tissue Culture Services Ltd (Botolph Claydon, UK). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from Sigma Chemical Co Ltd, Poole, UK. Triton X-100 was purchased from BDH Ltd, Poole, UK. Dulbecco phosphate buffered saline (DPBS) was made using PBS tablets (Oxoid Basingstoke UK) which contained supplements of $CaCl_2$ (0.1 mg/ml) and $MgCl_2$ (0.1 mg/ml). Human pituitary growth hormone (IS 80/505) was provided by the National Institute for Biological Standards and Control, South Mimms, UK. The assigned specific bioactivity of this reference preparation is 2.6 U/mg.

2.2. Manufacture of cement

PMMA (CMW-3) cement was produced by adding methyl methacrylate (5 ml) to the polymer powder (10 g). This was carried out using the proportions recommended by the manufacturers. LHBC was made by adding the monomer n-butyl methacrylate (5.5 ml) to the polymer powder (10 g). For the hGH-loaded cements, 12 IU of hGH was added to 10 g of the appropriate polymer powder, followed by careful mixing (10 min) to ensure homogeneity, before addition of the appropriate liquid monomer. The cements were allowed to dough and then cast into discs in polyethylene moulds (diameter 15 mm and 4 mm deep). Immediately after completion of the polymerization the discs were weighed.

2.3. Release studies

Both hGH-loaded and unloaded control discs of PMMA and LHBC were eluted into 5 ml of the bioassay medium which is described below. This was accomplished by incubation on a rollermixer, at 37 °C. The eluate was removed at selected times and replaced with fresh medium prewarmed to 37 °C. The samples were either assayed immediately or snap frozen in liquid nitrogen and used after storage at − 20 °C.

2.4. Cells

Nb2 rat lymphoma cells were routinely grown in suspension culture in growth medium which consisted of a basic RPMI-1640 medium which contained 50 U Penicillin/ml, 50 µg streptomycin/ml, 2×10^{-3} M L-glutamine, and serum additions. For growth medium the additions were fetal calf serum (FCS) (10%) and horse serum (10%). The cells were incubated in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Stocks of cells were cultured in 25 cm^2 tissue culture flasks and split twice per week with alternate 1:5 and 1:10 dilutions.

2.5. Bioassay

Prior to bioassay the cells were transferred to a quiescent medium for 24 h to halt cell proliferation. Quiescent medium contained 1% FCS instead of the 10% FCS used for the growth medium. For the bioassays the cells were resuspended in bioassay medium which consisted of the basic RPMI-1640 medium + 10% horse serum only. The cells were dispensed into microtitre plates (50 µl), at a density of 8×10^5 cells/ml. This was followed by the addition of the appropriate standard or sample (50 µl), which was also diluted in bioassay medium.

In the bioassay, the cells were exposed to hGH standards or eluates from the release studies for a total of 96 h. After this the colorimetric reagent MTT (5 mg/3 ml DPBS), was added to each well (30 µl), and the incubation continued (60 min) in a dry incubator at 37 °C. Acidified Triton X-100 (30 µl; 16% Triton X-100, 0.1 M HCl) was added to solubilize the formazan produced by the metabolic reduction of MTT, and the microtitre plates were gently shaken (30 min). Optical densities (OD) were determined with a Biorad microtitre plate reader (Model 3550, Richmond, CA) at a measurement wavelength of 595 nm and a reference wavelength of 655 nm.

With this ESTA bioassay, within-assay percentage error in hormone measurement, i.e. imprecision, of less than 10% can be achieved between 0.03 and 2.5 mU hGH/1, with the optimal precision of 2.5% being obtained at 0.5 mU hGH/1. The between-assay imprecision for 12 independent bioassays which were run

over a period of 6 months, each of which had a separate serial dilution of the hGH standard, was found to be 11% and 9% at 0.078 and 0.312 mU hGH/1, respectively.

2.6. Assessment of cytotoxicity

The ESTA system was adapted to assess the potential cytotoxicity of the eluates from LHBC and PMMA discs as follows. Eluates obtained from cement discs which had not been loaded with hGH, were added to Nb2 cells. These cells were simultaneously activated by hGH (0.625 mU/1). Controls contained assay medium in place of the eluate. At the end of a 96 h exposure period, the colorimetric end-point of the ESTA bioassay was determined as described above. The results for each eluate were expressed as a percentage of the response of the activated Nb2 cells in the controls (100%) in which assay medium had been substituted for the eluates. When appropriate, changes in cell number were also determined in parallel, using a Coulter Counter (Coulter Electronics Inc, Hialeah, FL).

2.7. Immunoassay

The immunoassays for hGH, were performed using an immunoradiometric assay (IRMA) based on reagents provided by NETRIA (London, UK). This is a monoclonal–polyclonal solid phase IRMA. The standard used is IS 80/505, which is identical to the one used in the ESTA bioassay. The within-assay percentage errors in hormone measurement, i.e. imprecision, were 5.1%, 2.4% and 2.6% at hGH concentrations of 0.8, 4.5 and 86.5 mU/1, respectively, while between-assay imprecision was 3.5%, 5.2% and 5.5% at hGH concentrations of 7.7, 21.7 and 45.8 mU/1, respectively [17].

2.8. Data analysis

The results from both the bioassays and the immunoassays were processed using the 1224 Multicalc Immunoassay programme (Pharmacia, UK) which also assessed within-assay imprecision. Statistical evaluations were performed using Minitab 8 (Minitab Inc, PA).

3. Results

3.1. The cytotoxic effects of eluates from unloaded LHBC and PMMA

When hGH-activated Nb2 cells were exposed to neat eluate from LHBC discs for 96 h, their response to the hormone was almost completely suppressed (Fig. 1). The discs had been eluted for 24 h. No viable cells were detected upon visual inspection of the microtitre plate wells, after exposure of the cells to the neat eluate. In contrast, the neat eluate from PMMA suppressed the response of the Nb2 cells to hGH by only 44%. In addition, we found that the cytotoxicity of the neat eluates from both cements greatly decreased for eluates obtained after those harvested at 24 h (data not shown). This indicated that the toxicity was due to a component which predominantly leached from the cement during the first 24 h.

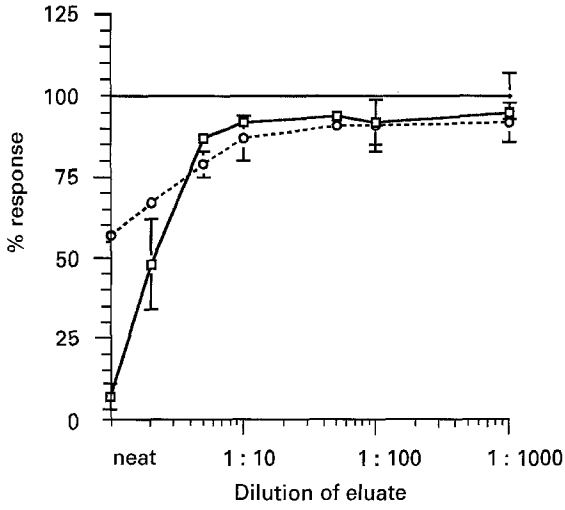

Figure 1 The effect of the eluates from unloaded PMMA and LHBC upon the response of Nb2 cells to hGH. The eluates from cement discs that had been eluted for 24 h were diluted sequentially to a final dilution of 1 : 1000. These eluates (50 µl) were added to the wells of a microtitre plate containing Nb2 cells (8×10^5 cells/ml) and hGH (0.625 mU/l) in a volume of 50 µl. These were then incubated (96 h) and the colorimetric end-point of the ESTA bioassay was determined by the addition of MTT, as described in Section 2. Triplicate wells were used, and the results are shown as means ± SD (LHBC (□), PMMA (○)). The results are the responses obtained in the presence of the eluate expressed as a percentage of the response obtained in the controls when the eluates were replaced with bioassay medium. The latter is taken to be 100%. The responses were initially obtained as equivalent concentrations of hGH as measured from a standard hormone dose–response curve which was run in parallel. The serial dilutions for each cement were tested in one microtitre plate.

The cytotoxicity of the eluates decreased upon dilution (Fig. 1). The cytotoxic effect of LHBC upon Nb2 cells is rapidly diluted out, such that suppression of the response of the hormone-activated cells is halved by a 1:2 dilution, and is virtually abolished by a 1:10 dilution. In contrast with PMMA the plateau is approached more gradually. The diluted eluates approach the controls, such that they are no longer significantly different from the control values.

These cytotoxic properties of the unloaded cements could have introduced artifacts into our later experiments in which we measure the potency of hGH on Nb2 cells, after the release of hormone from the cements, However, in these later experiments, we were able to dilute out these cytotoxic effects. This was confirmed by our finding that when we spiked LHBC eluates which had been diluted in assay medium (1:100), with hGH (0.625 mU/1) satisfactory recoveries were obtained (98 ± 5%, $n = 3 \pm$ SD) .

3.2. The effect of storage on the cytotoxicity of unloaded LHBC and PMMA discs and their eluates

If release of the residual monomer from the cements is responsible for their cytotoxicity, these effects would

TABLE I The effect of storage on the cytotoxicity of discs of unloaded LHBC and PMMA and of their 24 h eluates

Additive	% response: + LHBC eluate	% response: + PMMA eluate
Fresh eluate from unstored discs	No detectable response	65 ± 3
Stored eluate (24 h; RT) from unstored discs	No detectable response	85.3 ± 4
Eluates from stored discs (RT)		
24 h storage	45.7 ± 7	85.7 ± 4
72 h storage	53 ± 3	91 ± 9
168 h storage	56.4 ± 2	ND
Eluate from stored discs (37 °C)		
168 h storage	68.3 ± 2	ND

Neat eluates (50 µl) obtained after elution for 24 h from discs of LHBC and PMMA, which had not been loaded with hGH, were added to the wells of a microtitre plate containing Nb2 cells (8×10^5 cells/ml) and hGH (0.625 mU/l) in a volume of 50 µl. These were then incubated for 96 h and the colorimetric end-point of the ESTA bioassay was determined. Triplicate wells were used, and the results are shown as means ± SD. These results are the response obtained in the presence of the eluate, expressed as a percentage of the response obtained in the absence of the eluate, which is taken to be 100%. The responses were initially obtained as equivalent concentrations of hGH as measured from a standard hormone dose–response curve which was run in parallel. The discs and their eluates had been subjected to different storage conditions as detailed in Table I. RT = room temperature, ND = not determined.

be expected to decrease if the discs or the eluates are stored at room temperature, because these monomers are volatile. Storage of the eluates from freshly prepared PMMA discs for 24 h at room temperature prior to their addition to the hormone activated Nb2 cells, decreased their cytotoxicity. This treatment increased responses of the Nb2 cells such that 85.3 ± 4%, as opposed to 65 ± 3% of the response of the fully activated cells was obtained (Table I). In contrast, the cytotoxicity of the eluates from LHBC discs was not decreased by similar storage of the eluates. This confirmed the enhanced cytotoxicity of the eluates from LHBC as opposed to PMMA discs. However, we found that storage of LHBC discs, as opposed to eluates, at room temperature for extended periods of time (24–168 h) greatly reduced the cytotoxicity of their subsequent eluates. For example storage for 1 week (168 h) resulted in the response of the Nb2 cells increasing from an undetectable level to 56.4 ± 2% of the control. Eluates obtained from PMMA discs which had been stored for 72 h at room temperature were no longer significantly cytotoxic. These results suggest that the cytotoxicity of the eluates was at least in part due to a volatile component which evaporated on storage. This would be consistent with our finding (Table I) that if the LHBC discs were stored at 37 °C as opposed to room temperature, the cytotoxicity of the eluates obtained from them was further reduced. After this treatment, 68.3 ± 2% of the response of the controls was achieved.

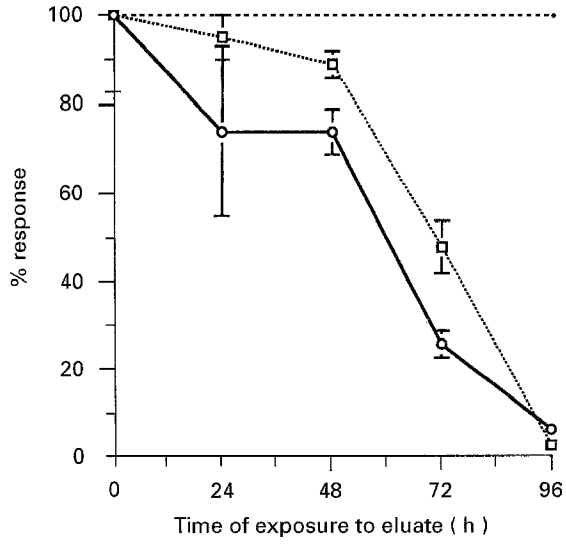

Figure 2 The effect of time of addition of the eluate from unloaded LHBC upon the response of Nb2 cells to hGH. Neat eluate (50 µl) was added to the wells of a microtitre plate containing Nb2 cells (8×10^5 cells/ml) and hGH (0.625 mU/l) in a volume of 50 µl. These eluates were either added simultaneously with the hormone or after delays of selected times. The final eluate addition occurred immediately prior to the determination of the colorimetric end-point (□), or counting the cells (○). Controls with (50 µl) bioassay medium in place of the eluates were also added to parallel wells at the same times as eluate addition. Triplicate wells were used throughout, and the results are the responses obtained in the presence of the eluate expressed as a percentage of the responses obtained in the fully activated appropriate controls. The latter were taken to be 100%. The results for both the colorimetric end-points and also the cell counts are expressed as means ± SD. For the ESTA system, the responses were initially obtained as equivalent concentrations of hGH as measured from a standard hormone dose–response curve which was run in parallel.

3.3. The effect of the exposure of Nb2 cells to the neat eluate from unloaded LHBC for different time intervals

For the neat eluate from LHBC to completely suppress the response of the Nb2 cells to the hormone, we found that a full 96 h exposure time was necessary (Fig. 2). As previously stated, when the neat eluate was added at the start of the bioassay, giving a full exposure time of 96 h, the response of the cells to hGH was almost virtually abolished. However, when the Nb2 cells were incubated with the eluate for shorter time intervals, its inhibitory effects on the responses of the Nb2 cells to growth hormone were decreased. Thus the response was increased to 48 ± 6% of the controls when the cells were exposed to the eluate for only 72 h. A concomitant reduction in cell killing with decreasing exposure times was observed. Thus the results in the ESTA bioassay were paralleled when cell numbers were monitored, the total abolition of response in the bioassay after a 96 h exposure time being associated with a very low cell count.

3.4. The release of bioactive growth hormone over time: PMMA versus LHBC

Over 90% of the bioactive hGH released from both LHBC and PMMA occurred in the first 24 h of elution (Fig. 3a) when respective recoveries of 0.58 ± 0.06% and 0.59 ± 0.04% of the hGH initially

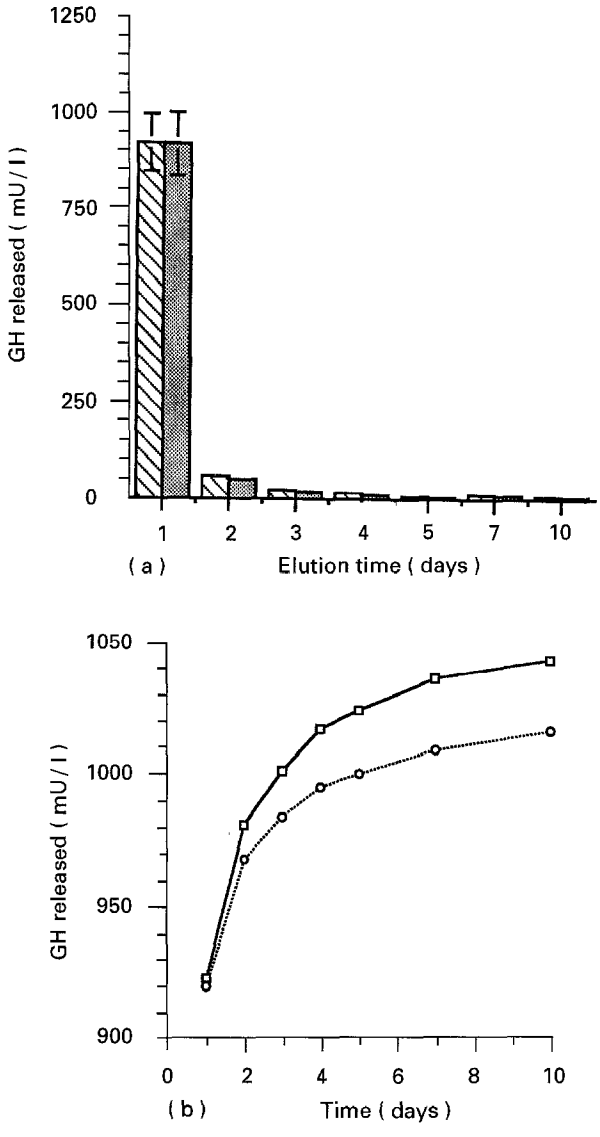

Figure 3 The release of hGH from PMMA and LHBC followed for prolonged elution times. Individual discs of LHBC and PMMA were eluted into assay medium (5 ml), and the eluate sampled at regular intervals for up to 10 days. These eluates were then stored at − 20 °C until measured in the ESTA bioassay. The results are expressed as hGH released as a function of increasing elution time (Fig. 3a) LHBC (▨) and PMMA (▩). Any cytotoxicity due to the cements themselves has been abolished by diluting the eluates prior to their analysis. Triplicate discs were eluted individually into the 5 ml volumes of the assay medium, and the results are expressed as means ± SD. The cumulative release of hGH with increasing elution times is depicted in Fig. 3b (LHBC (□) and PMMA (○)), as calculated from the results shown Fig. 3a. The results are expressed as the summed means obtained from the individual discs of each cement at each time point.

incorporated into the cements were obtained. From the elution profile it appears that there was no significant difference in the release of hGH from the two cements. However, when plotted as cumulative release (Fig. 3b), LHBC appeared to release slightly more hGH than PMMA. It will be noted that an apparent equilibrium is reached at about 10 days. Given the dimensions of the discs, this would suggest a diffusion coefficient of 10^{-7} to 10^{-8} $cm^2 s^{-1}$. This is higher than the diffusion coefficient of water in PMMA [18], and similar to that in the LHBC [7], indicating that the release process is complex.

3.5. Release of hGH after 24 h elution: bioactivity versus immunoactivity

In a separate experiment the bioactive concentrations of hGH in the eluates from the multiple discs of the two cements (24 h elution) were again found to be not significantly different (Table II). However, for both cements, the immunoactive concentrations of the hGH in these eluates were notably higher than those determined by the bioassay ($p = < 0.0001$). As a consequence the recoveries of immunoactivity as opposed to bioactivity were also greater. In addition we found that there were significant differences in the amounts of the hormone released from the two cements when these were measured by immunoassays, (LHBC, 1920 ± 424 versus PMMA, 1494 ± 118 mU/l; $p = < 0.05$). Most importantly the ratio of the bio: immunoactivity (B:I ratio) significantly declined for the eluates obtained from both cements, from a starting value before incorporation of 0.75 ± 0.06, to lower values of 0.62 ± 0.04 (PMMA ($p = < 0.0001$)) and 0.47 ± 0.05 (LHBC ($p = < 0.0001$)) after release from the cements. The greater decrease in the B: I ratio for LHBC suggests that the structural integrity of the hGH after its release from LHBC is more compromised than that released from PMMA. An alternative explanation, namely that the cytotoxicity of LHBC was introducing an artifact into the bioassay such that the bioactivity of eluates from this cement was negatively biased, did not apply. This is because the determination of the bioactivities in the experiment were made on samples which had been extensively diluted (1:1000), at which dilutions the cytotoxic effects are diluted out (Fig. 1). In confirmation of this, we noted that parallelism was retained when hGH was

TABLE II A comparison of the release of bioactive and immunoactive growth hormone from LHBC and PMMA.

	Bioactivity hGH (mU/l)	% recovery	Immunoactivity hGH (mU/l)	% recovery	B:I
LHBC	896 ± 187^{af}	0.62 ± 0.16^{b}	1920 ± 424^{cf}	1.34 ± 0.33^{d}	0.47 ± 0.05^{e}
PMMA	925 ± 91^{ag}	0.61 ± 0.09^{b}	1494 ± 118^{cg}	0.91 ± 0.10^{d}	0.62 ± 0.04^{e}

^{a,b} = not significant, $^{c,d}p = < 0.05$, $^{e,f,g}p = < 0.0001$

Multiple discs of each growth hormone loaded cement ($n = 9$ (LHBC); $n = 10$ (PMMA)) were eluted (24 h) individually into assay medium (5 ml). Samples of each were immediately analysed in the bioassay, after a dilution of 1:1000. Aliquots of the eluate that had been stored (2 months; − 20 °C) were also analysed in the immunoassay, at a dilution of 1:50. The results are expressed as concentrations of hGH in the eluate, which are means for the individual discs ± SD. The amount of hGH released has also been calculated as a percentage of the hormone initially added to individual discs, i.e. as weight corrected percentage recoveries. The means of these percentage recoveries (± SD) for each group are also shown. The significance of the differences observed between unpaired variables when tested by Students' *t*-test are indicated (a–g).

measured in eluates from hGH loaded LHBC which were bioassayed at dilutions of 1:1000 and 1:10000. We also noted that there was a larger variation in the concentrations of hGH in the eluates obtained form the nine LHBC discs than from the 10 PMMA discs when measured by both bio- and immunoassay.

4. Discussion

Revell *et al.* [10] concluded that LHBC was less toxic than PMMA from *in vivo* studies. However, paradoxically the detailed studies possible with our *in vitro* bioassay system revealed that neat eluate from LHBC suppressed the response of activated Nb2 cells more markedly than those from PMMA. In addition, cell counting demonstrated that these cytotoxic effects were due to cell killing. Our further studies showed that the cytotoxicity decreased when the discs were stored. This suggested that it was largely due to a volatile component. For LHBC this could be the monomer, n-butyl methacrylate, which was used in its formulation. This conclusion is consistent with our finding that storage of PMMA was especially effective in reducing the cytotoxicity of this biomaterial (Table I). This could be because the monomer used for PMMA, methylmethacrylate, has a boiling point which is 60 °C lower than that of the n-butyl methacrylate, used for LHBC, and would consequently evaporate more readily.

Comparisons between the toxic effects of the two monomers, as opposed to the cements, have yielded conflicting reports. Revell *et al.* [8] found that n-butyl methacrylate was slightly more toxic than methylmethacrylate when injected into mice. However, early studies by Mir *et al.* [9] reported that n-butyl methacrylate was less toxic to isolated perfused rabbit hearts. This apparent conflict may have been due to differences in the clearance rates of the monomers, which will influence *in vivo* but not *in vitro* studies. The clearance rate of n-butyl methacrylate has not been reported, but methylmethacrylate is cleared in humans within minutes [19].

The cytotoxic effects of eluates from LHBC were only manifested in our *in vitro* system when exposure times were long, e.g. 2–4 days. This was evident from both the colorimetric and cell counting experiments. These cytotoxic effects may therefore have only limited significance for *in vivo* systems. For the subsequent studies on the bioactivity of the hGH released from the cements, when the bioassay was run over a 96 h period, it was important that these toxic effects were eliminated. This was readily achieved by dilution of the eluates (1:1000) prior to analysis.

LHBC and PMMA release similar amounts of bioactive hGH after 24 h elution. Although only ~0.6% of the hormone which was originally incorporated into the discs is released, this is sufficient to provide concentrations in the eluates (~900 mU/l) which are high relative to concentrations of hGH normally expected in the circulation (0.5–20 mU/l). Significantly greater amounts of immunoactive hGH were released from both cements and LHBC released more immunoactive hGH than PMMA.

Direct comparison between two assays, such as the NETRIA immunoassay and the ESTA bioassay, allows one to use these two systems to reflect the structural integrity of complex proteins such a hGH. Any change in B:I ratios indicates changes in the structure which are influencing bio- and immunoactivity of the molecule differentially. Moreover, immunoassays have been reported to be the least discriminating technique for assessing the integrity of hGH [20]. The B:I ratio of the native hormone, before its incorporation into the cements was 0.75 ± 0.06, and we note that after incorporation into PMMA and LHBC it decreased to 0.62 ± 0.04 and 0.47 ± 0.05, respectively. Since both cements released similar quantities of bioactive hGH, one interpretation of this is that LHBC in particular released structurally compromised hGH, which was bioinactive but fully immunoactive. This is the first report of the use of two assays to assess the structural integrity of hGH after its incorporation in this way. Our finding that the B:I ratio of the hormone before its incorporation was 0.75 ± 0.06, i.e. less than unity, despite the use of the same pituitary standard (IS 80/505) in both the bio- and immunoassays, must reflect differential recognition of this heterogeneous standard which was derived from pituitaries, relative to the recombinant Austrophin, which was used for the incorporation studies, in the two assay systems. It may be linked to the positive bias previously reported for the NETRIA assay [17].

During incorporation into an orthopaedic bone cement hGH is exposed to elevated temperatures and the organic components of the cement. Since the heat of polymerization for LHBC (50–60 °C) is lower than that for PMMA (80–90 °C) [4], less thermal damage to the hormone would be expected with LHBC. One of the components, namely benzoyl peroxide, which initiates polymerization, and is included in the formulation of both of the cements, may be potentially damaging to the hGH molecule. As little as 0.1% and 0.01% hydrogen peroxide has been reported to produce severe and mild oxidation of the hormone, respectively [20]. However, although from our studies, the structure of hGH after release from LHBC appeared to be the more compromised than that for PMMA, slightly less benzoyl peroxide (1.8%) was used in the formulation of LHBC compared to PMMA, which required 2.0%. It is possible, however, that more free peroxide was available within the LHBC discs.

In conclusion, no difference was observed between the amounts of bioactive hGH released from the two substantially different orthopaedic bone cements investigated in this study. However, LHBC released a significantly larger amount of immunoactive hGH. The undiluted eluates from both unloaded LHBC and PMMA proved to be significantly cytotoxic in our *in vitro* system. This was more marked for LHBC, and was probably largely attributable to the toxicity of the volatile monomers used in the formulation of these cements.

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References

1. B. D. MULLIKEN and R. B. BOURNE, *Current Opinion in Orthopaedics* **5** (1994) 11.
2. L. LINDER, in "Biocompatibility of orthopaedic implants", Vol. II, edited by D. F. Williams (CRC Press, Boca Raton, Florida, 1982) pp. 1–23.
3. W. D. SCHWARZ and M. BRADEN, *J. Dent.* **1** (1973) 179.
4. M. BRADEN, R. L. CLARKE, G. J. PEARSON and W. CAMPBELL KEYS, *Brit. Dent. J.* **141** (1976) 269.
5. B. WEIGHTMAN, M. A. R. FREEMAN, P. A. REVELL, M. BRADEN, B. E. J. ALBRETSSON and L. V. CARLSON, *J. Bone Jt. Surg. (Brit)* **69** (1987) 558.
6. M. P. PATEL and M. BRADEN, in "Failure of joint replacement. A biological, mechanical or surgical problem", edited by S. Downes and M. Dabestani (The Institute of Orthopaedics Stanmore, UK, 1994) pp. 13–16.
7. K. W. M. DAVY and M. BRADEN, *Biomaterials* **12** (1991) 540.
8. P. REVELL, M. GEORGE, M. BRADEN, M. FREEMAN and B. WEIGHTMAN, *J. Mater. Sci. Mater. Med.* **3** (1992) 84.
9. G. N. MIR, W. H. LAWRENCE and J. AUTIAN, *J. Pharm. Sci.* **62** (1973) 778.
10. P. REVELL, M. BRADEN, B. WEIGHTMAN and M. FREEMAN, *Clin. Mater.* **10** (1992) 233.
11. S. DOWNES, D. J. WOOD, A. J. MALCOLM and S. Y. ALI, *Clin. Orthop. Rel. Res.* **252** (1990) 294.
12. J. W. PRITCHETT, *Acta. Orthop. Scand.* **63** (1992) 520.
13. S. DOWNES, *Clin. Mater.* **7** (1991) 227.
14. P. A. EALEY, M. E. YATEMAN, S. J. HOLT and N. J. MARSHALL, *J. Mol. Endocrinol.* **1** (1988) R1.
15. P. A. EALEY, M. E. YATEMAN, R. SANDHU, M. DATTANI, M. HASSAN, S. HOLT and N. MARSHALL, "Growth regulation" **5** (1994) 36.
16. T. TANAKA, R. P. C. SHIU, P. W. GOUT, C. T. BEER, R. L. NOBLE and H. G. FRIESEN, *J. Clin. Endocrinol. Metab.* **51** (1980) 1058.
17. P. J. PRINGLE, J. JONES, P. C. HINDMARSH, M. A. PREECE and C. G. D. BROOK, *Clin. Chem.* **38** (1992) 553.
18. G. D. STAFFORD and M. BRADEN, *J. Dent. Res.* **47** (1968) 341
19. B. GENTIL, C. PAUGAM, C. WOLF, A. LIENHART and B. AUGEREAU, *Clin. Orthop. Rel. Res.* **287** (1993) 112.
20. A. F. BRISTOW and S. L. JEFFCOATE, *Biologicals* **20** (1992) 221.

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