Effect of PMMA cement radical polymerisation on the inflammatory response

MATTEO SANTIN*
School of Pharmacy and Biomolecular Sciences, University of Brighton, Lewes Road, Brighton BN2 4GJ, UK

ANTONELLA MOTTA
Dipartimento di Ingegneria dei Materiali, Universita' di Trento, Via Mesiano 77, 38050 Trento, Italy

ASSUNTA BORZACHIELLO, LUIGI NICOLAIS, LUIGI AMBROSIO
Istituto dei Materiali Compositi e Biomedicali, Consiglio Nazionale delle Ricerche, Naples, Piazzale Tecchio 80, 80153 Naples, Italy

The effect of the radical polymerisation taking place during the hardening of the polymethyl methacrylate (PMMA) bone cements is known to cause bone necrosis through the relatively high exothermic reaction and the leaching of toxic non reacted monomers. The inflammatory response towards this class of cements has also been shown and ascribed mainly to the phagocytosis of the material particles. However, the effect of the radical polymerisation on the adsorption of plasma proteins and on the activation of monocytes/macrophages when the material is in a non-phagocytosable dimension has not been elucidated.

In the present work, the polymerisation of three bone cements, CMW-1, Palavit and Simplex-P in a clinically reflective environment and its effect on the formation of a surface conditioning film as well as on the inflammatory cell activation were investigated.

The data showed that on CMW and Simplex-P the polymerisation was not fully accomplished. CMW released high levels of non-reacted monomers, no significant macrophage adhesion and high oxidative burst and cytokine production. The relatively lower levels of released monomers in Simplex and Palavit seemed to promote a lower inflammatory response while cell adhesion was favoured by patches of plasma components entrapped in the hardening dough during the polymerisation.

Introduction

Although new bioactive cements based on calcium phosphate pastes have been developed in the last few years [1], poly(methyl methacrylate) (PMMA)-based cements are still the materials of choice to anchor arthroplasties to the contiguous bone [2]. Since the introduction of these type of materials by Charnley [3] in the early sixties many commercial products have been developed [4]. The hardening of the polymerising paste at the site of the implantation, however, is cause of many drawbacks which ultimately lead to a limited performance of the implant. The highly exothermic reaction and the release of non-reacted monomers are the two main problems associated with the use of the PMMA cements [2, 4]. The consequent necrosis of the bone tissue contacted by the cement becomes a weak zone prone to fracture under the continuous mechanical stress to which the implant/bone construct undergoes [2].

A further cause of concern in the use of the PMMA cements is the inflammatory response triggered by these materials. A consistent body of data has been published where the inflammatory response to PMMA cements has been evaluated *in vitro* and *in vivo* [5–8]. In particular, the efforts of many research groups have been focused onto the inflammatory response generated by the release of cement particles being originated from the shear stress between cement and bone [2]. *In vitro* studies have been performed to evaluate the activation of the monocytes/macrophages induced by PMMA particles [5, 6]. The size of the particles seems to be an important factor, the phagocytosed debries being particularly strong activators of the cells. However, it has also been outlined that the erosion of the bone surrounding the cement seems to lead to the aseptic loosening of the construct only when in combination with other factors such as the interfacial mechanical failure, the bond failure, the bone remodelling, and the cement failure suggesting that the inflammatory response dictated by the cement particles

*Author to whom all correspondence should be addressed.

play only a limited role in the failure of the implant [2, 5].

Few investigations have taken into account the effect of the radical polymerisation on the humoral and cell components of the inflammatory response [7]. The present paper aims to study the activation of the humoral components and the activation of the monocytes/macrophages induced by the radical polymerisation of acrylic cements in absence of phagocytosis. Clinically reflective experimental models were adopted in the attempt of highlight the effect of human plasma in the host response to polymerising cement paste.

Materials and methods

Cement preparation

A 24-well plate for cell culture (Nunc, Milan, Italy) was prepared by coating the bottom of the wells with discs of polyacetate sheet. Five hundred microlitres of the incubation media were added to each well. The incubation media chosen were either freshly isolated human plasma or phosphate buffer saline (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl) pH 7.4 (PBS). Bone cement discs (1.0 ± 0.2 cm diameter, 1-mm thickness) were prepared from three commercial products, CMW-1 (CMW Lab, Dentsply, UK), Simplex-P (Howmedica, London, UK) and Palavit (Hereus Kulzer, Werheim, Germany). The pastes were prepared according to the manufacturers' instructions. The cement dough was prepared according to the classical hand-mixing procedure using a spatula. Immediately after mixing an aliquot of the paste was poured into each well and shaped with the spatula in form of disk. The discs were kept in the incubation medium (plasma or PBS) for 15 min at room temperature to allow complete hardening. The discs were then washed twice in PBS for 5 min at room temperature and incubated in 0.5 ml of PBS for 1 h at 37 °C. By this type of procedure two kinds of specimens were prepared for each cement: (i) control cement discs (CMW-C, Pal-C and Simp-C) and (ii) cement discs conditioned with plasma during the polymerisation (CMW-P-P, Pal-P-P, Simp-P-P). A third type of specimen was then prepared by incubating a disc polymerised in PBS in 0.5 ml of human plasma for 1 h at 37 °C (CMW-P-T, Pal-P-T, Simp-P-T).

The heat released by the three types of cements at the interface with the incubation medium (PBS) during polymerisation was detected by a thermocouple and the temperature values expressed as peak polymerisation temperature in Celsius degrees.

The discs were washed three times in PBS and used for the experiments.

Surface scanning electron microscopy (SEM)

The different types of bone cement discs were freeze-dried overnight, sputter-coated by gold and analysed by SEM (Leica Stereoscan 440, Cambridge, UK) at 5 keV, at different magnification. The analysis was performed in duplicate.

Differential scanning calorimetry (DSC) of the bone cement discs prepared in PBS

The thermal properties of the cements were studied by differential scanning calorimetry using a differential scanning calorimeter (DuPont mod.2100). The samples of about 10 mg were obtained from the relative control discs. Dynamic scans at a rate of 10 °C/min from 0 to 180 °C were performed.

Monomer release

The release of non-reacted species from the material bulk was followed on the extract obtained from the washes in PBS and from the incubation of the discs in PBS for 1 h at room temperature. The optimal reading in the ultraviolet region of the spectrum was assessed by scanning 1 ml of PBS solution containing an aliquot of each commercial liquid monomer (5 µl). The spectrum was obtained after blanking the instrument against PBS. Once the optimal reading was assessed standard curves of the monomer of each commercial preparation were obtained in the range $0.25–0.97 \times 10^{-4}$ mg/ml. Standard curves with linearity coefficients of 0.899, 0.943 and 0.859 were obtained for CMW, Simplex and Palavit, respectively. The experiments were performed in triplicate on samples prepared on different days.

Monocytes/macrophages activation and adhesion

The activation of the monocytes/macrophages was assessed on mononuclear cells freshly isolated from human peripheral blood by Boyum's method [9].

The oxidative burst of the cells was evaluated by chemiluminescence by a method previously described [10]. Briefly, 10^5 cells were added to a vial containing the tested cement discs immersed in a PBS medium containing 0.1 µM Luminol (Sigma, Milan, Italy) to a final volume of 3 ml. The chemiluminescence was detected by a Packard 1600TR (Packard, Milan, Italy) scintillation spectrometer. Each sample was read every 5 min for 0.25 min. The spontaneous activation of the cells in absence of cement disc was also evaluated. The peak count per minute values were taken and data expressed as CPM mean $\pm$ standard deviation from $n = 6$. Data were statistically analysed by t-test and samples considered significantly different at $p < 0.05$.

The morphology of the adhering monocytes/macrophages on PMMA discs treated with human plasma during or after radical polymerisation was analysed by SEM. The cells were seeded at 10^5/ml in RPMI-1640 medium for 3 h at 37 °C, in 95% air, 5% CO_2 environment. The discs were extensively washed in PBS, fixed in 2.5% (w/v) glutaraldehyde in PBS, and step wise dehydrated in ethanol. The specimens were finally freeze-dried overnight, sputter-coated by gold and analysed by SEM at 5 keV.

The activation of the cells at longer term was evaluated as release of interleukin-1beta (IL-1β) in the culture medium. The cells were cultured as reported above for 24 h and the supernatants withdrawn, centrifuged at 1000 × to remove possible non-adhering cells and stored at −70 °C until use. One hundred

microlitres of each supernatant were analysed for the concentration of IL-1β by an Amersham kit (Milan, Italy) and data expressed as mean (pg/ml) ± standard deviation from $n = 6$. Tissue culture plates (TCP) were used as control.

The viability of the mononuclear cells in presence of disc extracts was evaluated by Trypan blue exclusion method. The cells (2.5×10^5) were seeded in presence of 2 ml of the extraction medium (RPMI-1640 supplemented with 10% fetal calf serum) used for the third washing step of the discs after their polymerisation in PBS. A positive control was prepared by adding 0.1 ml of Triton X-100 to the growth medium. After 24 h incubation, the cells were washed in PBS and stained in 0.04% (w/v) Trypan blue (Sigma, Milan, Italy) solution in PBS. The viable cells were scored at 20 × magnification by an inverse phase light microscope (Hund Wezlar, Germany) from five random fields. The experiments were performed in duplicate on samples and controls.

Results

The analysis of the surface morphology clearly showed that in PBS CMW paste preserved a compact morphology, whereas Simplex and Palavit presented different degrees of porosity (Fig. 1(a)–(c)). The radical polymerisation in the presence of human plasma also showed differences from one type of cement to another (Fig. 2(a)–(d)). CMW showed the deposition of filamentous structures (Fig. 2(a) and (b)), whereas Simplex was almost completely covered by a granular layer of organic material (Fig. 2(c)). Palavit did not show any visible deposition of plasma components (Fig. 2(d)), its surface being not significantly different from the cement prepared in PBS (Fig. 1(c)).

Differences in the thermal properties and the polymerisation behaviour of the three types of cements were also detected. The peak polymerisation temperatures, obtained by the thermocouples, for CMW, Simplex and Palavit were 32.0, 36.2 and 25.7 °C, respectively.

The thermal analysis of the three cements prepared in PBS showed a peak of exothermic reaction for CMW and Simplex during the first scan (data not shown). The (ΔH_{res}) values for these reactions were 25.54 J/g for the CMW and 16.13 J/g for Simplex (Table I). As expected, the exothermic peak did not appear in the second scan. In the first scan Palavit did not show any exothermic reaction. The different ΔH_{res} values indicate an amount of non-reacted monomers in CMW larger than Simplex and Palavit. The glass transition temperature, measured for the three materials at the second scan showed values of 97.47, 100.75 and 100.66 °C for CMW, Simplex and Palavit, respectively (Table I).

Table II shows the mean concentration values of the leachates from the three cements measured by the spectrophotometric method. The analysis of the third PBS wash showed that CMW released a concentration of monomers approximately four times higher than Palavit and 10 times higher than Simplex. Similarly, after 1 h incubation of the discs in PBS CMW still released monomer concentration approximately four times higher than Simplex and six times higher than Palavit.

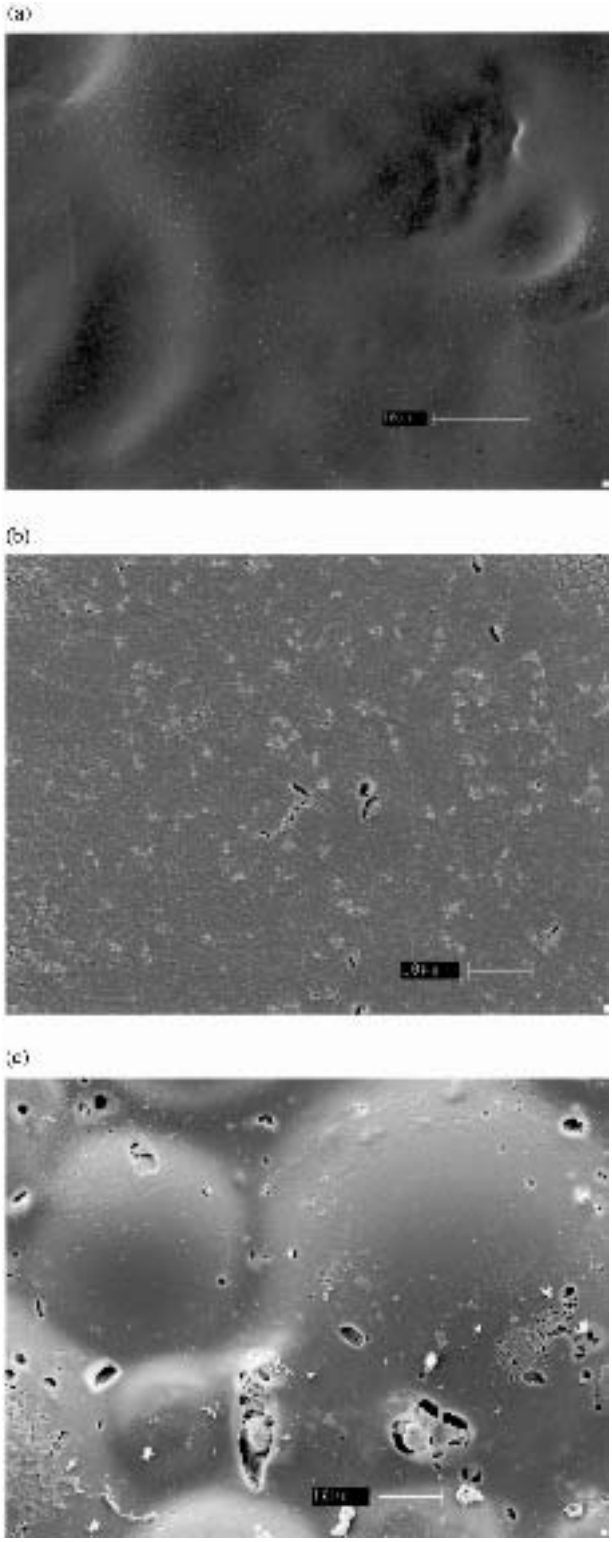

Figure 1 SEM of the bone cement surface morphology after polymerisation in PBS. (a) CMW, (b) Simplex, (c) Palavit.

A relatively higher oxidative burst by the monocytes/macrophages was induced by CMW regardless its surface conditioning method (Fig. 3). Simplex and Palavit showed a monocyte oxidative burst not significantly higher than the control cell sample. Only Palavit showed a slight significant increase when its surface was treated with human plasma after polymerisation.

Similarly, the cells showed a significant production of IL-1β after 24 h incubation only when incubated on the surface of CMW discs treated with human plasma during or after polymerisation (Table III). CMW polymerised in

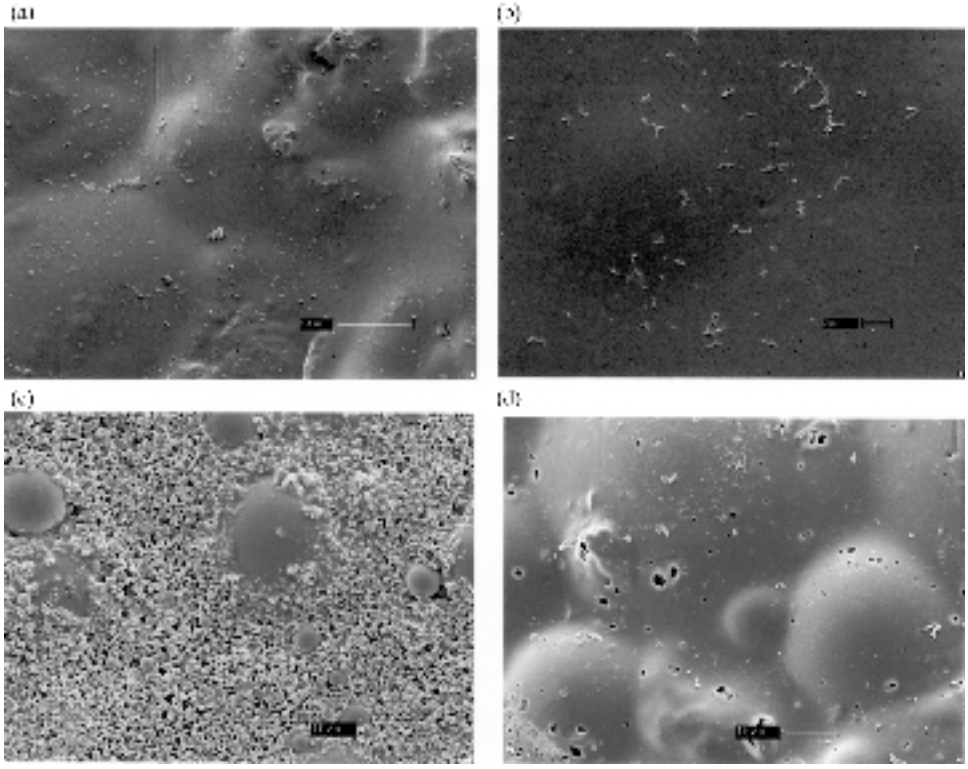

Figure 2 SEM of the bone cement surface morphology after polymerization in human plasma, (a and b) CMW, (c) Simplex, (d) Palavit.

TABLE I Thermal properties of the cements

	ΔH_{res} (J/g)	T_g (°C)
CMW-C	25.54	97.47
Simp-C	16.13	100.75
Pal-C	—	100.66

TABLE II Released monomer concentration from the PMMA cements

Sample ($n = 3$)	Leachate concentration mean [10^{-4} mg/ml] (range)	
	3rd PBS wash	After 1 h in PBS
CMW-C	26.24(22.56–29.91)	31.37(28.40–34.35)
Simp-C	2.80(1.76–3.83)	8.46 (6.98–9.93)
Pal-C	6.84(5.71–7.98)	5.68 (4.74–6.61)

TABLE III Cement-induced IL-1β production by the monocytes/macrophages

Sample ($n = 6$)	IL-1β (pg/ml) ± standard deviation
TCP	858.6 ± 91.3
CMW-C	4.2 ± 5.94
CMW-P-P	11.3 ± 2.8
CMW-P-T	18.5 ± 1.4
Simp-C	—
Simp-P-P	5.7 ± 0.8
Simp-P-T	2.8 ± 2.3
Pal-C	1.7 ± 1.6
Pal-P-T	—
Pal-P-P	—

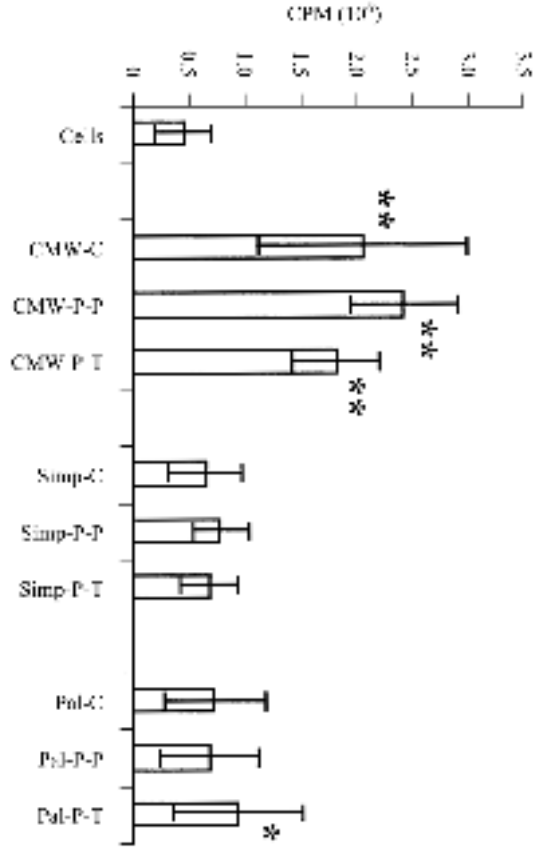

Figure 3 Monocyte oxidative stress induced by the PMMA cements. * Indicates sample significantly different from the control cells at $p < 0.05$. ** Indicates sample significantly different from the control cells at $p < 0.01$.

PBS as well as the other two types of cements without and with human plasma pre-conditioning did not show significant production of this cytokine.

The analysis of the morphology of the adhering cells showed no significant adhesion (data not shown). Only few, round-shaped cells were observed on Simplex

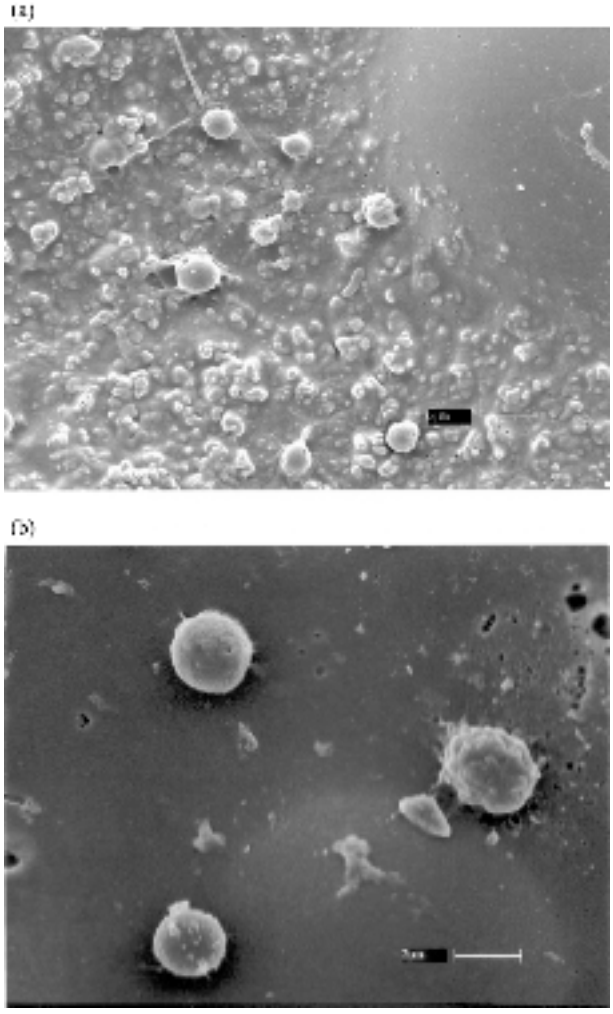

Figure 4 SEM of monocytes/macrophages adhering on PMMA cement surfaces, (a) Simp-P-P, (b) Pal-P-P.

polymerised in the presence of plasma, the cells adhering preferentially on the granular organic material deposited during the paste hardening (Fig. 4(a)). Palavit was the only cement where a significant number of adhering cells were found (Fig. 4(b)). However, these cells were round-shaped and characterised by a smooth surface.

The viability of the cells, evaluated as integrity of their cell membrane by Trypan blue, was not significantly affected by the disc extracts of the three cements the score being not significantly different from the negative control and among samples (data not shown).

Discussion

The majority of the articles published on the effect of the PMMA-based cements on biological components concern investigations about their toxicity and their inflammatory potential on the surrounding tissues [11–13]. Clinical studies have provided information about the late effects of these cements on the bone after several years of implantation [14–16]. Studies *in vitro* have also contributed to clarify many aspects of these toxic and inflammatory processes [5, 6]. However, no study has been performed to elucidate the effect of the radical polymerisation on the formation of a film of plasma proteins on the surface of the cements. This layer of proteins inevitably forms on the surface of the cement when it is injected in the bleeding bone tissue. The role of adsorbed proteins in determining the biocompatibility of the implants is widely recognised because of the receptor role of these proteins for tissue and inflammatory cells [17]. It is also known that the change of conformation of the protein molecule during the adsorption process may elicit an immune response, the denatured proteins being regarded as foreign by the immune system. In the case of the PMMA-based cements these change of conformation may be accentuated by the exothermic reaction and by the formation of highly reactive species such as the free radicals of the polymerising paste. The three cements analysed in this paper were chosen on the basis of their completely different polymerisation temperature as reported by the manufacturer. CMW-1 is reported to have a peak polymerisation temperature of 120 °C, Simplex-P 100 °C and Palavit 45 °C. However, at the interface with the bleeding bone tissue the temperatures detected were far below the protein denaturation temperature (approximately 45 °C) ruling out any effect of the temperature in causing protein denaturation in the adopted experimental model. However, the analysis of the morphology showed that both CMW and Simplex were able to incorporate in their polymerising paste material originated from plasma although at different extents. Palavit seemed to be more inert than the other two cements towards interactions with the overlying plasma environment. In addition a different behaviour of the inflammatory cells on the three materials when treated with plasma during or after their polymerisation would suggest a different surface conditioning protein film. Protein studies seemed to support this hypothesis showing different levels of adsorption for proteins key in the host response (data not shown).

Bulk polymerisation of methacrylate is strongly influenced by the monomer diffusion. The decreased diffusion of the monomers in the hardening paste reduces their chances of reaction with the available reactive centres thus leading to the presence of non-reacted monomers in the hardened material [18, 19]. The presence of non-reacted monomers can be detected by DSC. The residual heat of reaction (ΔH_{res}) detected during the first scan is indeed proportional to the amount of residual monomer in the polymerised material, while the second scan allows the determination of the glass transition temperature on the material cured during the first scansion. The DSC and monomer release experiments clearly showed that the polymerisation of both CMW and Simplex was not completed unless a curing step was performed, while the polymerisation of Palavit was spontaneously accomplished. These data, in combination with the SEM analysis, may suggest that the unaccomplished polymerisation of CMW and Simplex may involve the adsorbing plasma proteins which are thus embedded in the polymerising dough of these two cements.

The relatively high levels of monomer release may also affect the early cell-mediated inflammatory response. The production of free radicals by the cells as well as their degree of adhesion and cytokine secretion seem to be dependent on the levels of release of monomers by the cement bulk. The monocytes would be stimulated by these non-reacted monomers to produce

an early oxidative burst, but they will be soon inactivated loosing their ability to bind the material surface and to produce cytokines. Their viability seemed to be not impaired by the monomers when analysed by Trypan blue. However, this test evaluates only the integrity of the cell membrane. Indeed, it has been proven that hydrophobic MMA monomers are able to penetrate the cell membrane without inducing any damage of the membrane, but being toxic for the cell [11, 20].

Conclusions

The effect of the monomer leaching from PMMA cements may alter the normal inflammatory response thus interfering on the normal wound healing process. Both the aseptic and septic loosening of the cemented prostheses may be therefore triggered by a subtle action of the non-reacted monomers eliciting, respectively, the immune response towards denatured adsorbed proteins or inhibiting the protective role of the macrophages towards infections.

References

1. E. J. HARPER, *J. Eng. Med.* **212** (1998) 113.
2. G. LEWIS, *J. Biomed. Mater. Res.* **38** (1997) 155.
3. J. CHARNLEY, *J. Bone. Joint Surg.* **43B** (1960) 28.
4. K. D. KUHN, in ''Bone Cements'' (Springer-Verlag, Berlin, 2000) p. 7.
5. O. GONZALES, R. L. SMITH and S. B. GOODMAN, *J. Biomed. Mater. Res.* **30** (1996) 463.
6. W. MICHELL, J. B. MATTHEWS, M. H. STONE, J. FISHER and E. INGHAM, *Biomaterials* **24** (2003) 737.
7. L. A. THOMSON, F. C. LAW, K. H. JAMES, C. A. MATTHEW and N. RUSHTON, *ibid.* **13** (1992) 811.
8. N. ISHIGURO, T. KOJIMA, T. ITO, S. SAGA, H. ANMA, K. KUROKOUCHI, Y. IWAHORI, T. IWASE, and H. IWATA *J. Biomed. Mater. Res.* **35** (1997) 399.
9. A. BOYUM, *Scand. J. Clin. Lab. Invest.* **21** (1968) 77.
10. M. SANTIN, A. MOTTA, G. FREDDI and M. CANNAS, *J. Biomed. Mater. Res.* **46** (1999) 382.
11. G. CIAPETTI, D. GRANCHI, E. CENNI, L. SAVARINO, D. CAVADEGNA and A. PIZZOFERRATO, *ibid.* **52** (2000) 338.
12. G. ZAMBONIN, S. COLUCCI, F. CANTATORE and M. GRANO, *Calcif. Tissue Intern.* **62** (1998) 362.
13. J. CHIBA, L. J. SCHWENDEMAN, R. E. BOOTH, S. CROSSET and H. E. RBASH, *Clin. Orthop. Rel. Res.* **299** (1994) 114.
14. S. B. GOODMAN, *J. Long Term Effect Med. Impl.* **6** (1996) 97.
15. S. B. GOODMAN, P. HUIE, Y. SONG, K. LEE, A. DOSHI, B. RUSHDIEH, S. WOOLSON, W. MALONEY, D. SHURMAN and R. SIBLEY, *J. Clin. Orthop. Rel. Res.* **337** (1997) 149.
16. T. P. HARRIGAN, J. A. KAREH, D. O. O'Connor, D. W. BURKE and W. H. HARRIS, *J. Orthop. Res.* **10** (1992) 134.
17. T. L. BONFIELD, E. COLTON and J. M. ANDERSON, *J. Biomed. Mater. Res.* **26** (1992) 457.
18. A. BORZACCHIELLO, L. AMBROSIO, L. NICOLAIS, E. J. HARPER, K. E. TANNER and W. BONFIELD, *J. Mater. Sci.: Mater. Med.* **9** (1998) 317.
19. A. BORZACCHIELLO, L. AMBROSIO, L. NICOLAIS, E. J. HARPER, K. E. TANNER and W. BONFIELD, *ibid.* **9** (1998) 835.
20. D. GRANCHI, S. STEA, G. CIAPETTI, L. SAVARINO, D. CAVEDAGNA and A. PIZZOFERRATO, *Biomaterials* **16** (1995) 1187.

Received 6 February
and accepted 30 June 2004