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Effects of phosphate-based glasses on T lymphocytes *in vitro*

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Host responses to implanted materials can lead to the production of inflammatory mediators and thereby induce potentially adverse reactions, including the activation of T lymphocytes. Although such cells are a central component of immune reactions and likely to be fundamental in determining the long-term clinical efficacy of implants, their response to biomaterials is not well known. This study has therefore examined the *in vitro* effects of phosphate-based glasses (PG), which can be produced with pre-determined solubility and may be promising materials for promoting the regeneration of new bone and other tissues. Extracts of PG which were modified by the addition of Ca, Co, Zn, and Fe oxides were found to cause only very low levels of activation of human peripheral blood T lymphocytes over a period of 6 days, as measured by changes in DNA synthesis. In contrast, the activation of these cells by concanavalin A, a potent T cell mitogen, was partially inhibited by extracts of a high-Ca PG and nearly totally ablated by the Co-derived extract. These studies show that, despite their apparent inability to activate immunologically responsive cells directly, substances which leach out of metal-containing PG implant materials have the potential to modulate inflammatory reactions.
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1. Introduction

The biocompatibility of implant materials is of major concern in orthopaedic and dental surgery. Following implantation, host-material interactions occurring at the implant site can induce a potentially adverse inflammatory reaction [1]. The infiltration of polymorphonuclear leukocytes and macrophages leads to the production of inflammatory mediators and the activation of T lymphocytes. Such cells are pivotal in the immune response and thus likely to be fundamental in determining the long-term clinical success of implanted materials.

Calcium phosphate-based glasses (PG) and ceramics are considered to have potential value as biomaterials for the repair and regeneration of damaged tissue [2]. They consist of a basic system in which phosphorous pentoxide (P_2O_5) is the network former and sodium oxide (Na_2O) and calcium oxide (CaO) are the network modifiers. Such PG have been used as bone substitutes because of their chemical similarity with bone [3]. They can also be formulated to specified degrees of solubility. Moreover, PG glasses can be modified by the addition of oxides such as CoO, ZnO, NiO, and Fe_2O_3, which change their mechanical, physical and chemical properties and have also been shown to modulate their bioactivity [4–6]. For example, Kawamura *et al*. [7] have demonstrated that the zinc-containing calcium phosphate implants enhanced the formation of bone in rabbits compared with control implants without zinc.

Reactions to metal ions can also sometimes produce deleterious effects, including those mediated by immune and non-specific inflammatory responses [8]. For example, *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) and T lymphocytes by metal ions has been reported to lead to strong proliferative responses in allergic individuals, with evidence of T cell involvement in the pathogenesis of metal hypersensitivity [9]. Other studies of elevated levels of metal ions in the joints and sera of patients who have undergone implant surgery suggest that these may be sufficient to impair immune cell function [8]. Moreover, the presence of implant-derived metal-containing wear debris has been shown to promote the activation of macrophages, leading to the production of inflammatory mediators [10] and modulating local inflammatory activity [11]. Little is

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TABLE I Composition of quaternary PG glasses

Glasses	Composition (mol %)			
	Metal	CaO	Na_2O	P_2O_5
Ca40	—	40	15	45
CoO	5	35	15	45
ZnO	5	35	15	45
Fe_2O_3	5	35	15	45

known, however, about the effects of biomaterials, and particularly metal-containing materials such as PG, which have a certain degree of solubility, on lymphocytes and lymphocyte activation. The present study was therefore carried out: (i) to determine the ability of metal-doped PG to directly activate T lymphocytes and (ii) to measure the effects of the PG on T cell activation by concanavalin A (Con A), a polyclonal T cell mitogen.

2. Materials and methods

2.1. PG composition and preparation

The glasses used in this study are based on a quaternary system consisting of fixed P_2O_5 (45 mol %), Na_2O (15 mol %) and CaO (35 mol %), with the remaining 5% of either calcium oxide (CaO), cobalt oxide (CoO), zinc oxide (ZnO) or iron oxide (Fe_2O_3), as shown in Table I. The appropriate amounts of the solid compounds were combined, heated at 1000–1200 °C for 1 h in a Pt/10% Rh crucible (Type 71040) and poured into graphite molds at 300–400 °C. After cooling slowly to room temperature, the remelted PG rods were cut into discs 12 mm in diameter and 2 mm thick. They were sterilized by dry heat at 180 °C for 2 h.

2.2. Preparation of PG extracts

''Extracts'' of the PG were prepared by placing the discs in 10 ml of cell culture medium (see below) for 5 days at 37 °C, after which the extract was collected, filtered through a 2 μm filter and stored at −20 °C. Dilutions of 1 : 2 (50%) and 1 : 10 (10%) of the glass extracts were prepared in culture medium immediately prior to use.

2.3. Preparation of T lymphocytes

Normal human peripheral blood was obtained from healthy volunteers, with informed consent as approved by the Joint Research and Ethics Committee of the Eastman Dental Institute and Hospital. The blood was diluted 1 : 1 with the full culture medium (see below), layered onto Ficoll and centrifuged at 400 g for 25 min at room temperature. The lymphocyte/monocyte interface layer was collected and washed twice with phosphate-buffered saline (PBS) by centrifugation at 800 g for 5 min and placed into 75 cm^2 culture flasks containing 5 ml of full RPMI medium (supplemented with 10% foetal calf serum and 1% penicillin, streptomycin and L-glutamine). After 2 h at 37 °C to allow the monocytes to adhere, the non-adherent T lymphocytes were removed and used as described below.

2.4. Effects of PG extracts

In order to examine whether the PG extracts could activate resting lymphocytes, replicate aliquots of 0.1 ml of a suspension containing 10^6 cells/ml were placed into 96-well plates and incubated in the presence of 10% and 50% of each of the PG extracts. Control flasks contained medium only, with no added PG extract. The activation of the T cells was monitored by measuring changes in DNA synthesis. This was carried out by adding [^{3}H] thymidine (1 μCi/ml; 37 Ci/mmol) (Amersham, UK) for 2 h at 37 °C on days 1, 2, 3 and 5 of culture. The cell suspensions were harvested on filter papers (Whatman No. 1), washed with 5% trichloroacetic acid (TCA) at 4 °C and the precipitated radiolabelled DNA measured in a scintillation counter.

To measure the effects of the PG extracts on T cells activated by the polyclonal T cell mitogen Con A (Sigma, Aldrich), replicate cell cultures containing 10 μg/ml Con A were incubated in the presence of the PG extracts and DNA synthesis measured as above, on days 1, 2, 3, 5 and 6 of culture. Control wells contained medium and mitogen only, with no glass extract.

3. Results

3.1. Effects of PG extracts on DNA synthesis by T lymphocytes

Incubation of T cells with all of the PG extracts resulted in increased levels of DNA synthesis compared with control cultures which had no added PG extract, as shown in Fig. 1. Although the overall pattern of DNA synthesis was the same in all cultures, being maximal on day 3, the level of DNA synthesis differed between the different PG extracts. Thus, while both the Ca40- and Co-containing PG extracts increased DNA synthesis by approximately 70%, the Zn- and Fe-containing cultures elicited an increase of approximately 100% compared with that on days 1 and 2. It is of interest that DNA synthesis in the presence of the extracts of the Zn-containing PG was substantially reduced at day 2 compared with control, as also observed at day 1 (data not shown). Notably, both the profile and extent of T cell activation remained the same when the extracts were diluted from 1 : 2 to 1 : 10 (Fig. 1(A) and (B), respectively), including the apparent inhibitory effect of the Zn-PG at day 2. Despite these differential effects of the PG extracts, DNA synthesis in the control and all of the test cultures was very low compared with cultures incubated with the polyclonal mitogen Con A, as described below.

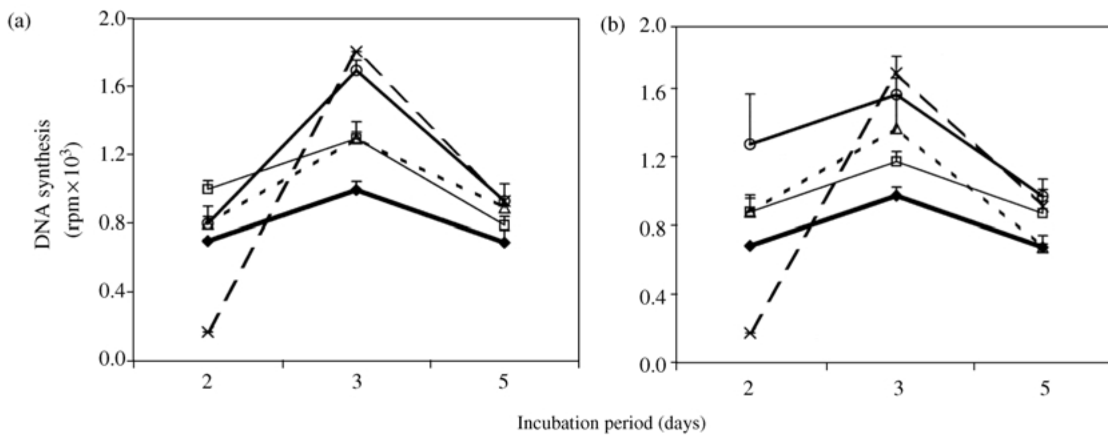

Figure 1 Effects of PG extracts on DNA synthesis by T lymphocytes. Changes in DNA synthesis by resting T lymphocytes incubated in the presence of (A) concentrated and (B) diluted PG extracts, as a measure of T cell activation. Data are the mean ± SEM dpm. Control cultures were T cells with medium and no glass extract (◆—◆, control; □—□, Ca40; △—△, Co; x– –x, Zn; ○—○, Fe). DNA synthesis on day 1 was the same as on day 2 (data not shown).

3.2. Effects of PG extracts on Con A-induced T cell activation

Many previous studies have shown that there is a marked increase in DNA synthesis by resting T cells in the presence of Con A [12, 13]. In the present study it was also found that DNA synthesis over a period of 6 days reached a level that was approximately 20-fold greater than at day 2 (Fig. 2). A very similar high level of T cell activation by day 6 was observed when the cells were cultured with the Fe- and Zn-containing PG extracts, but incubation of the resting T cells with the concentrated Ca40-containing PG extract resulted in approximately 50% inhibition of DNA synthesis (Fig. 2(a)). However, when the extract was diluted (1 : 10), Con A-induced T cell activation reached the same high level as in control cultures (Fig. 2(b)). It is notable, however, that extracts derived from the Co-containing PG had a marked inhibitory effect, reducing DNA synthesis by more than 90% (Fig. 2(a)), to the same low level observed in cultures of resting T lymphocytes with no Con A (Fig. 1). Moreover, even the diluted Co extract was found to block T cell activation (Fig. 2(b)). Overall, other than with the Ca-derived extract, incubation of T cells with the other resulted in no apparent differences in DNA synthesis between the concentrated and diluted extracts.

4. Discussion and conclusions

PG glasses are considered to have potential value for bone regrowth in orthopaedic and dental surgery and also for the regeneration of soft connective tissues. However, in the presence of biological fluids, components of the ''soluble'' glasses are likely to leach out into the surrounding tissue and into the blood and these could have deleterious as well as beneficial effects. Thus, it is of importance to determine the precise biological response to substances which may be released from PG materials. The present study has therefore examined the effects of extracts of metal-doped PG on T lymphocytes and the T cell activation process.

The current study has shown that most of the PG extracts were able to directly cause activation of the resting T lymphocytes, as seen by the increased levels of DNA synthesis compared to control cultures. Moreover, there were differential stimulatory effects of the extracts, since Zn- and Fe-containing PG appeared to be more active than these containing Ca and Co. In addition, these effects were even maintained after the extracts were diluted. However, these effects may be of only limited biological consequences as DNA synthesis induced by the extracts was very low compared with the very high level of activation elicited by the well-known polyclonal mitogen Con A.

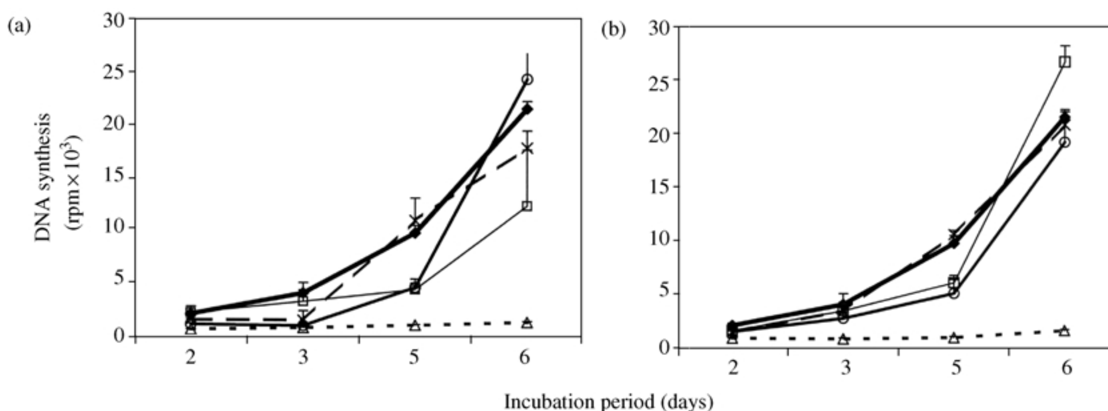

Figure 2 Effects of PG extracts on Con A-induced T cell activation. DNA synthesis by Con A-activated T lymphocytes incubated with (a) concentrated and (b) diluted PG extracts. Data are the mean ± SEM dpm. Control was cells and medium with mitogen, containing no glass extract (◆—◆, control; □—□, Ca40; △—△, Co; x– –x, Zn; ○—○, Fe). DNA synthesis on day 1 was the same as on day 2 (data not shown).

Despite the apparent stimulation of DNA synthesis by the resting lymphocytes, most of the extracts had little, if any, detectable effect on the level of T cell activation elicited by Con A. Although the Ca- and Fe-containing extracts appeared to exert some inhibition, this was either transient or abolished by dilution of the extract. However, there was a persistent and marked inhibitory effect on T cell activation in the presence of the Co-containing PG extract. This is consistent with recent studies which have shown that Co ions also inhibited phytohemagglutinin- and lipopolysaccharide-induced T cell activation and the release of IL-2, IL-6 and IFN-γ [8], and also that the presence of Co ions released from metal alloys is associated with reduced numbers of lymphocytes [14]. It is notable, however, that despite the marked inhibitory effect of Co-PG on the Con A-induced activation of T the cells, the present study also found that Co did not prevent the low level of ''constitutive'' activation of the cells. Indeed, in T cells cultured without mitogen, Co appeared to enhance DNA synthesis (Fig. 1).

Our findings suggest that metal-doped PG have the capacity to influence the activity of T lymphocytes. Studies are now in progress to identify the specific components released into the PG extracts and to determine the functional effects on specific types of T cells.

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