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Development and characterization of interleukin-18-loaded biodegradable microspheres

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Abstract

Immunostimulation represents a promising approach designed to specifically eradicate malignant cells. Since glioma tumour cells hole up in the central nervous system (CNS) in a particularly inauspicious milieu to antitumour immune reactions we here propose a new strategy to revert the properties of this microenvironment by administering an antitumour cytokine into the CNS tumour itself. Thus, biodegradable poly(D,L-lactide-*co*-glycolide) (PLGA) sustained-release microspheres for stereotaxic implantation loaded with interleukin-18 (IL-18), that is known to exert antitumour activity and trigger immune cell-mediated cytotoxicity, were developed. Different tests for assessing IL-18 bioactivity were set-up and evaluated. A specific bioassay was considered as the most reliable test. The stability and integrity of IL-18 was then verified during the encapsulation process. Consequently, two procedures of IL-18 encapsulation in PLGA microparticles (W/O/W and S/O/W) were investigated. As determined by radiolabelling studies using ^{125}I-IL-18 and a continuous flow system, the in vitro release profile of IL-18 was optimum with S/O/W method with a moderate burst effect and a subsequent progressive discharge of 16.5 ± 8.4 ng/day during the next 21 days against 6.1 ± 4.2 ng/day with the W/O/W method. Considering analytical testing of IL-18 together with its preserved biological activity after release from microspheres, amounts of the active cytokine obtained with S/O/W method were relevant to plan in vivo evaluation to validate the therapeutic strategy.

Keywords: Cancer therapy; Adjuvants; Controlled drug delivery systems; Cytokine release; Poly(lactic-*co*-glycolic acid); Interleukin-18; Microspheres

1. Introduction

Although the central nervous system (CNS) can be involved in crosstalks with the immune system and is able to ensure by itself several immunomodulatory functions (Xiao and Link, 1998) uncovering limits to its historical "immune privilege" (Barker and Billingham, 1977), this environment remains particularly inauspicious to antitumour immune reactions. Thus, immune-based treatments against tumours located in the CNS have generally not achieved the results seen for peripherally located tumours (Graf et al., 2003).

Those findings can be explained by the presence of the blood–brain barrier (BBB), the absence of conventional lymphatic draining, the weak level of expression of major histocompatibility complex (MHC) molecules and the poor amount of professional antigen presenting cells (APC). Moreover, the existence of mechanisms of apoptosis of activated immune cells (Weller et al., 1998; Didenko et al., 2002), the secretion of suppressive factors, such as "transforming growth factor-β" (TGF-β) (Weller and Fontana, 1995) which inhibits Th1 cell response and NK cell activity, and the presence of growth factors and neuronal activities that favour Th2 cell response, also support the lack of efficient antitumour immune reaction. In parallel, glioma themselves generate a wide range of cell death or immune escape phenomena such as the production of soluble decoy receptor 3 (Roth et al., 2001) or tenascin-C (Ruegg et al., 1989; Herold-Mende et al., 2002).

Since immunostimulation represents a promising approach designed to specifically eradicate malignant cells, a substan-

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tial collective effort has recently been made trying to revert unwanted immunosuppressive characteristics of tumour cells that hole up within the CNS. Hence, effective anti-CNS tumour immune responses have been generated by immune-based treatments such as adoptive T-cell transfer (Mukai et al., 1999; Quattrocchi et al., 1999); GAA-pulsed DCs (Ashley et al., 1997; Liau et al., 1999), and cytokine-secreting gliomas, fibroblasts, neural stem or mesenchymal stem cells (Sampson et al., 1996; Benedetti et al., 1999; Glick et al., 1999; Ehtesham et al., 2002; Graf et al., 2003; Nakamura et al., 2004) lending further credibility to the idea that the efficient induction of a cellular antitumour immune response can be targeted to antigens within the CNS.

If gene therapy represents an important hope, it also raises several problems, such as the stability of the transfection, the biological security and the long-term inocuity of viral vectors. Finally, the conceptual limit of this approach is that genetically modified cells may become themselves the target of the immune response. In addition, several antitumour cytokines have also displayed toxicity or represented a risk as a result of their involvement in certain neurological affections. This is the case of IL-2 which modifies BBB and induces an important cerebral oedema (Tjuvajev et al., 1995), and of TNF-α which is involved in the physiopathology of multiple sclerosis (Raine, 1995). Hence, the local delivery of bioactive proteins in the CNS still remains a major technological challenge.

In consequence, prolonged and controlled delivery of anticancer immunostimulatory cytokines by using particulate vectors would represent an interesting alternative. Biodegradable poly(D,L-lactide-*co*-glycolide) (PLGA) sustained-release microspheres have already been used for intracerebral implantation and could easily be implanted by stereotaxy in precise areas of the brain (Benoit et al., 2000). Moreover, PLGA, is a biocompatible copolymer to the brain tissue (Menei et al., 1993). Various drugs, especially therapeutic proteins like neurotrophic factors (nerve growth factor (NGF), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF)) have been encapsulated in this type of brain delivery system (Mittal et al., 1994; Maysinger et al., 1996; Pean et al., 1998; Aubert-Pouessel et al., 2004).

Interleukin-18 (IL-18), originally referred as interferon-γ-inducing factor (IGIF) was first described as an endotoxin-induced serum factor produced by spleen cells with the ability to stimulate the production of interferon-γ (IFN-γ) (Nakamura et al., 1989). Cloned in 1995, the IL-18 gene is transcribed/translated into a 24 kDa inactive peptide precursor known as pro-interleukin-18 (pro-IL-18) (Liu et al., 2000). Activation of 18.3–18.4 kDa mature IL-18 is initiated upon cleavage of pro-IL-18 by interleukin-1β converting enzyme (ICE) also referred to as caspase-1 (Gu et al., 1997). Functional IL-18 has been found to be produced by activated macrophage and dendritic cells. IL-18 also called IL-1F4, belongs to the IL-1β cytokine superfamily and interacts with a heterodimeric receptor complex (IL-18 receptor-α and IL-18 receptor-β) to mediate a wide range of immune and non-immune biological effects (for review, see (Dinarello, 1999; Golab, 2000). Apart stimulating IFN-γ production from macrophages, T cells and NK cells, IL-18 stimulates the production of IL-2, GM-CSF and chemokines (IL-8, MIP-1β and MCP-1) from peripheral blood mononuclear cells. IL-18 also promotes the development of Th1 cells from Th0 cells. IL-18 is able to enhance T and NK cell cytotoxicity towards tumour cells and exerts chemoattractive effects that have so far been demonstrated on Th1 T-lymphocytes (Komai-Koma et al., 2003), dendritic cells (Gutzmer et al., 2003) and NK cells (Ishida et al., 2004). Moreover, induction by IL-18 of the cell death factor FasL has also been observed on several cell types (Ohtsuki et al., 1997). IL-18 is also known to reduce angiogenesis in vivo, an effect which results in hypovascularization of treated tumours (Coughlin et al., 1998; Cao et al., 1999). Additionally, mice injected with an immunogenic tumour were able to reject it when treated with the cytokine (Osaki et al., 1998; Cao et al., 1999). Together, IL-18 broad activities support its interest for local CNS tumour immunotherapy.

Thus, the aim of the present study was to formulate PLGA microspheres that could be implanted stereotactically within the CNS and that could release in a controlled manner the active cytokine in accordance with the specifications for in vivo immunotherapeutic applications against gliomas. For that purpose, we investigated two procedures of IL-18 encapsulation in PLGA microparticles (W/O/W and S/O/W). A particular attention was given to the biological activity of the released protein that is a crucial point to validate new formulations.

2. Materials and methods

2.1. Materials

Rat recombinant IL-18, IL-2, IL-12, IFN-γ mouse recombinant IL-18, ELISA DuoSet antibodies directed against rat IFN-γ and human anti-CD28 were obtained from R&D Systems (Abingdon, UK). Rat anti-CD3 was purchased from BD Biosciences (Le Pont de Claix, France). PLGA was obtained from Boehringer Ingelheim (PLGA 25/50, Resomer RG 502, BI Chimie, Paris, France) and from Phusis (PLGA 37.5/25, Saint-Ismier, France). Bovine serum albumin (fraction V, 69 kDa), polyethyleneglycol 400 (PEG 400) and polyethyleneglycol 8000 (PEG 8000), DTT (Dithiothreitol), Sigmacote®, MOPS (3-[*N*-morpholino]propanesulfonic acid), EDTA and sodium azide, were from Sigma–Aldrich (Saint Quentin Fallavier, France). Sodium sulfate, sodium chloride, acetone, methylene chloride, polyvinyl alcohol (Rhodoviol 4/125, 88% hydrolyzed), were from Prolabo (Paris, France). Bolton-Hunter reagent (*N*-succinimidyl-3-(4-hydroxy-3-[^{125}I]-iodophenyl)propionate, monoiodinated, 250 μCi) was purchased from Perkin-Elmer Life Sciences (Courtaboeuf, France).

2.2. Analytical testing of IL-18 by SDS-PAGE and Western blotting

Carrier free IL-18 standard samples were analysed under reducing conditions on 12% polyacrylamide resolving gels by SDS-PAGE and Coomassie Blue staining. Western blots were performed as previously described (Aubert-Pouessel et al., 2004) using as primary antibodies directed against the rat recom-

binant IL-18 either a goat polyclonal antibody (0.1 μg/mL) or a mouse monoclonal antibody (1 μg/mL) (R&D Systems).

2.3. IL-18 enzyme-linked immunosorbant assay (ELISA)

ELISA was performed using immunoassay reagents provided by R&D Systems with washes in PBS pH 7.4 containing 0.05% Tween®20 between each step. ELISA plates (NUNC, Polylabo, Strasbourg, France) were coated by a mouse monoclonal IL-18 capture antibody (2 μg/mL) diluted in 0.1 M borate buffer pH 8.5. After overnight incubation at room temperature, plates were blocked for 1 h at room temperature in PBS pH 7.4 containing 1% BSA, 5% sucrose, before adding the samples appropriately diluted in PBS pH 7.4 containing 1% BSA. After 2 h incubation at 37 °C, bound IL-18 was detected by incubating for 1 h at 37 °C a biotin-conjugated goat polyclonal IL-18 recognition antibody (1 μg/mL in PBS pH 7.4 containing 1% BSA). Then, streptavidin-conjugated horseradish peroxydase (250 ng/mL in PBS pH 7.4 containing 1% BSA) was added to the plate for 1 h at room temperature. The enzyme substrate (tetramethylbenzidine and peroxydase) was added and incubated for 15 min. The enzyme reaction was stopped by adding acidic solution. Optical density was determined in a plate reader set at 450 nm and sample values calculated from the standard curve.

2.4. IL-18 bioassay

To assess biological activity of IL-18, a bioassay allowing the quantification of IFN-γ produced by IL-18 stimulated primary rat splenocytes enriched in T-lymphocytes was developed. Briefly, after gentle spleen mechanical dissociation, Ficoll purification and differential adhesion on plastic, isolated splenocytes were primed for activation during 24 h in dish previously coated for 1 h at 37 °C/5% CO_2 with 0.5 μg/mL anti-CD3 antibodies and in the presence of 0.3 μg/mL anti-CD28 in supernatant. They were further washed twice with Hank's balanced salt solution. Effects of IL-18 on the production of IFN-γ by isolated splenocytes, after 48 h stimulation in the presence of low amounts of IL-12 and IL-2 (0.1 and 0.4 ng/mL, respectively), were finally determined in supernatants by using a DuoSet ELISA system according to the manufacturer's instructions (R&D Systems).

2.5. PLGA coating

Fifty milligrams of PLGA 37.5/25 were dissolved in 2 mL of $CHCl_3$. The solution obtained was applied homogeneously onto Nunclon™ Δ polystyrene multidish surfaces (Nunc, Roskilde, Denmark) and evaporated for 1 h at room temperature. Ethanol 70° was then used during 45 min for sterilization of the coated dishes. After three washes in sterile water PLGA-coated multidish surfaces were kept under sterile tissue culture hood until use.

2.6. IL-18 ^{125}I-radiolabelling.

IL-18 was radiolabelled using ^{125}I-labelled Bolton Hunter's reagent (Perkin-Elmer, Paris, France). Briefly, 25 μg IL-18 were dissolved in 60 μL of MOPS buffer pH 7.2 (20 mM MOPS, 50 mM Na_2SO_4, 0.5 mM EDTA, 0.5 mM DTT) and added to a vial containing dried 250 μCi (9.25 MBq) Bolton Hunter's reagent. The reaction was performed at 4 °C with gentle vortexing for 1 h. ^{125}I-IL-18 was purified from the hydrolyzed products by gel filtration using a Sephadex PD-10 column (Amersham Biosciences, Orsay, France), which had been pre-equilibrated at 4 °C prior to use for 1 h with 0.5% gelatine solution and for an additional hour with MOPS buffer. ^{125}I-IL-18 was eluted with 4 mL MOPS buffer and 200 μL counted peak fractions were pooled.

2.7. Microsphere preparation

2.7.1. W/O/W method

Microspheres were produced according to a W/O/W emulsion solvent evaporation/extraction method (Aubert-Pouessel et al., 2004). For this purpose, a 150 μL internal aqueous phase consisting in 100 μL of MOPS buffer containing 3750 μg of BSA, 25 μg of IL-18 and a fraction of ^{125}I-IL-18, were mixed with 50 μL of PEG 400. The mixture was then emulsified in an organic solution (1.5 mL of methylene chloride and 0.5 mL of acetone) containing 50 mg of PLGA. The microspheres preparation was then pursued referring strictly to the previously published procedure.

2.7.2. S/O/W method

Protein-loaded microspheres were prepared accordingly to the S/O/W emulsion solvent evaporation method described by Morita et al. (2000) with minor modifications. Briefly, 1 mL of an aqueous mixture containing 125 μg of IL-18 (or ^{125}I-IL-18), 3.75 mg of BSA and 3.75 of PEG 8000 was lyophilized in coated glass tubes. Fifty milligrams of PLGA was directly weighed in the tube, where the protein–PEG aqueous mixture was lyophilized. After the addition of 1.5 mL of methylene chloride and 0.5 mL of acetone to the tube, the polymer was completely dissolved and the organic phase was completed. This organic phase was added into 30 mL of 5% (w/v) polyvinyl alcohol and 10% (w/v) NaCl aqueous solution, maintained at 1–2 °C and emulsified by mechanical stirring (Heidolph RGH 500, Prolabo, Paris, France) for 30 s at 550 rpm. The resulting emulsion was quickly poured into 400 mL of distilled water containing 10% (w/v) NaCl, and stirred for 25 min to extract the the organic solvent. The hardened microspheres were collected with a 0.45 μm meshed filter (HVLP type, Millipore SA, Maurepas, France), washed five times with 100 mL of deionized water and freeze-dried (RP2V Serail, SGD, Argenteuil, France) to obtain a free flowing powder. The dried particles were stored at +4 °C.

2.8. Optical microscopy studies, size determination and scanning electron microscopy (SEM) analysis

The microparticles were dispersed in a droplet of water, directly on a slide and immediately observed under optical microscopy (Olympus BH2, OSI, Paris, France) equipped with a video camera COHU and a computarized image analyzer (Microvision Instruments, Evry, France). The size determination was carried out using the computer software Granix 5.2.0

from Microvision Instruments. The surface and the internal morphology of the microspheres were investigated by scanning electron microscopy. Freeze-dried microspheres were mounted onto metal stubs using double sided adhesive tape, vacuum-coated with a carbon film (MED, Bal-Tec, Balzers, Lichtenstein) and directly analyzed under SEM (JSM 6301F, JEOL, Paris, France) equipped with a dispersive X-ray microanalysis apparatus (Link ISIS, Oxford, Orsay, France).

2.9. In vitro release study of IL-18 microspheres in a continuous flow system

In vitro release of IL-18 from PLGA microspheres was determined using a continuous flow system previously developed in our laboratory (Aubert-Pouessel et al., 2004). Briefly, a continuous flow is held upright by a syringe pump supplying MOPS buffer pH 7.2 (containing 0.1% BSA, 0.02% sodium azide) to the column inlet at 1 μL/min. Eluent was collected every 24 h in silanized tubes. The total amount of released protein was determined by counting radioactivity (cpm) with a gamma counter Cobra®II Packard Instruments (Paris, France). Samples were frozen at −20 °C until further analysis by bioassay.

3. Results and discussion

3.1. Analytical and functional testing of IL-18 integrity

In order to assess IL-18 integrity during each step of the formulation process and eventually when released from carrier systems, tests which are likely to be sensitive to the full range of differences which might result from the method change have been selected. They include analytical assays (SDS-PAGE, Western blot, ELISA) and in vitro bioassays. As shown in Fig. 1A, SDS-PAGE analysis of commercialized carrier-free IL-18 under reducing conditions accounted for protein size integrity and revealed a single band at the apparent molecular weight of 18.4 kDa. Since IL-18 amounts below 250 ng with a maximum possible loading volume of 100 μL were not detected, this method would not be suitable for concentrations lower than 2.5 μg/mL. Sequence and conformational preservation of the sale protein was evaluated by immunochemical methods. As shown of Fig. 1B, Western blot analysis under reducing conditions revealed a single band of the denatured protein at the apparent molecular weight of 18.4 kDa. While two different antibodies were tested in this assay and displayed distinct affinity, the best detection limit was 60 pg of IL-18 for 60 μL of loading volume meaning a concentration of 1000 pg/mL In contrast, the threshold of detection of IL-18 by ELISA reached 25 pg/mL. However, as shown in Fig. 1C and depending on the temperature this recognition was reduced with incubation time. While at 4 °C integrity was fully preserved during the first 3 h, protein conformation was altered at 20 °C after 3 h and quasi-totally lost at 37 °C after the same incubation time. The sensitivity and breadth of analytical testing is an important determinant of the nature and extent of additional testing which should be done. Therefore, biological integrity of IL-18 was evaluated in order to complement above analytical measurements. As stimulation of the production of IFN-γ is known to be one of the in vivo mech-

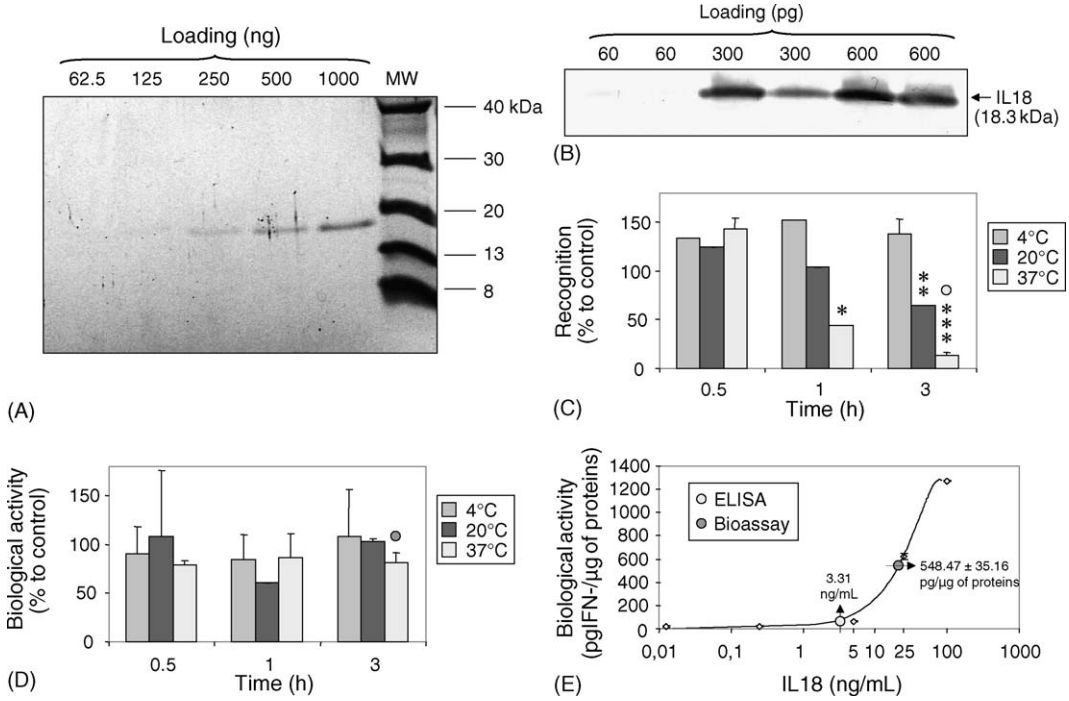

Fig. 1. Analytical and functional testing of IL-18 integrity. (A) SDS-PAGE and Coomassie blue staining. (B) Western blotting. (C) ELISA. (D) Bioassay. (E) Dose/response curve of standard IL-18 biological activity. Note the exponential increase of biological activity between 1 and 100 ng/mL IL-18. Note also that after 3 h incubation at 37 °C, of the initial 25 ng/mL IL-18 only 13.85%, meaning 3.31 ng/mL, are recognized (C and E, white dot). Interestingly, the corresponding biological activity determined with bioassay is 548.47 ± 35.16 pg IFN-γ/μg of proteins (D and E, gray dot), which remained close to the 100% control at 25 ng/mL IL-18. Student's *t*-test (differences from control values in C and D): $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.01$.

anisms of action of IL-18, a bioassay reflecting this activity was developed. Surprisingly, while recognition of IL-18 by ELISA was altered during time at 20 and 37 °C, no decrease in biological activity was observed in parallel (Fig. 1D). This discrepancy was not the reflection of the IL-18 concentration range that could trigger similar biological effects but truly the fact that IL-18 activity was preserved while epitope recognition by antibodies used during ELISA was lost. Indeed, after 3 h at 37 °C only 13.85% of the initial amount of IL-18 is detected which means a theoretical remaining concentration of 3.31 ng/mL for an initial concentration of 25 ng/mL. However, the corresponding biological activity observed experimentally (548.47 ± 35.16 pg/μg of proteins) remained close to the 100% at 25 ng/mL IL-18 and far much higher than the activity observed at 5 ng/mL IL-18 (Fig. 1E). As a consequence of the incongruity between IL-18 biological activity and immunochemical recognition, bioassays were used as the unique relevant assay for testing biological integrity during all the subsequent work made in the present study.

3.2. Effects of BSA and PEG on IL-18 biological activity

In order to determine whether a carrier protein such as BSA could influence IL-18 biological activity, bioassays using the rat recombinant cytokine from batches containing or not BSA were carried out. As shown in Fig. 2A, dose/response data demonstrate that the presence of 50 μg BSA for 1 μg IL-18 in original batches was crucial to keep optimal biological activity. As a consequence, the lack of BSA resulted in a reduction of at least a two-fold factor of IL-18 effects on the production of IFN-γ by splenocytes (Fig. 2A).

The role of the carrier protein on IL-18 biological activity was further analysed for possible interactions with PEG, a surfactant that usually serves for protein encapsulation in PLGA microspheres (Morita et al., 2000; Aubert-Pouessel et al., 2004). It has indeed already been shown that addition of PEG to an aqueous protein solution leads to preferential hydration of the protein, which can stabilize the native structure of globular proteins in aqueous solutions. As PEG concentration increases, the PEG can penetrate the hydration layer of the proteins, and the penetration leads to hydrophobic interaction between PEG and protein (Wang, 1999). For this purpose, effects of increasing concentrations of BSA combined or not with increasing concentrations of PEG were tested. As shown in Fig. 2B, minor changes on IL-18 activity were observed when BSA amounts varied from 50, then 87.5 to 425 μg for 1 μg IL-18. Similarly, increasing PEG concentrations from 0, then 37.5 to 375 μg for 1 μg IL-18 (combined or not with BSA) resulted in negligible effects on IL-18 activity. Thus, 50 μg BSA for 1 μg IL-18 is on own sufficient for optimal IL-18 activity.

The protective effect of BSA and other albumins (e.g. human or rat serum albumins) against protein unfolding and aggregation has been extensively documented and is likely due to their surface-active properties. Albumins are thought to occupy the interfaces and shield the therapeutic proteins from contact with the solvents or hydrophobic surfaces. As such several cytokines such as interferon-β (IFN-β) or interleukin-1 receptor antagonist (IL-1ra) that belongs to the IL-18 family displayed reduced activity when forming aggregates (Chang et al., 1996; Runkel et al., 1998). By contrast, significant protein protection was achieved when serum albumins were added to the inner aqueous phase during the primary emulsification step of multiple emulsion procedures (Bilati et al., 2005).

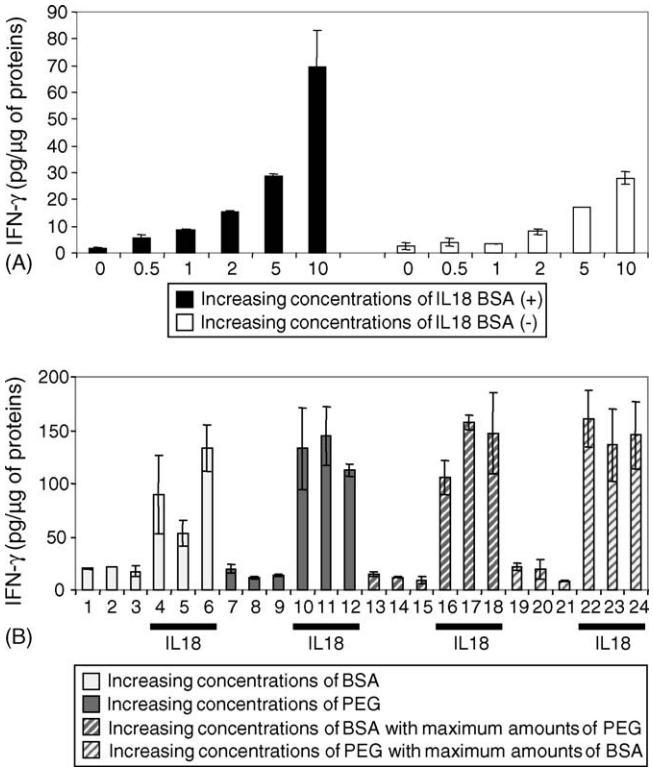

Fig. 2. Effects of BSA and PEG on IL-18 biological activity. (A) Dose/response histograms illustrating IL-18 activity with (+) or without (−) 50 μg BSA for 1 μg IL-18. IL-18 was used at concentrations ranged from 0 to 10 ng/mL (*x*-axis). (B) Conjugated effects of BSA and PEG. Treatments were made with MOPS buffer alone (1–3, 7–9, 13–15, 19–21) or IL-18 at the single dose of 10 ng/mL (4–6, 10–12, 16–18, 22–24). BSA concentrations varied from 50 (1, 4, 7–12, 13, 16), then 87.5 (2, 5, 14, 17) to 425 μg for 1 μg IL-18 (3, 6, 15, 18, 19–24). PEG concentrations varied from 0 (1–6, 7, 10, 19, 22), then 37.5 (8, 11, 20, 23) to 375 μg for 1 μg IL-18 (9, 12, 13–18, 21, 24).

3.3. Consequences of co-lyophilization on IL-18 biological activity

As we wanted to compare two separate formulation procedures for the preparation of PLGA microspheres and since one of them necessitates micronization that itself requires lyophilization steps, we tested IL-18 biological integrity after lyophilization in different conditions. As mentioned before, carrier free IL-18 displays strongly reduced activity when compared to our BSA containing control IL-18 (Fig. 3A and B). While the effects of lyophilization has minor consequences on IL-18 biological activity (not shown), co-lyophilization with BSA and PEG resulted in restoration of lost activity (Fig. 3C). Interestingly, while at low concentration effects of the micronized IL-18 were find to be higher than control IL-18, that was the opposite for high concentrations of the cytokine (Fig. 3A and C).

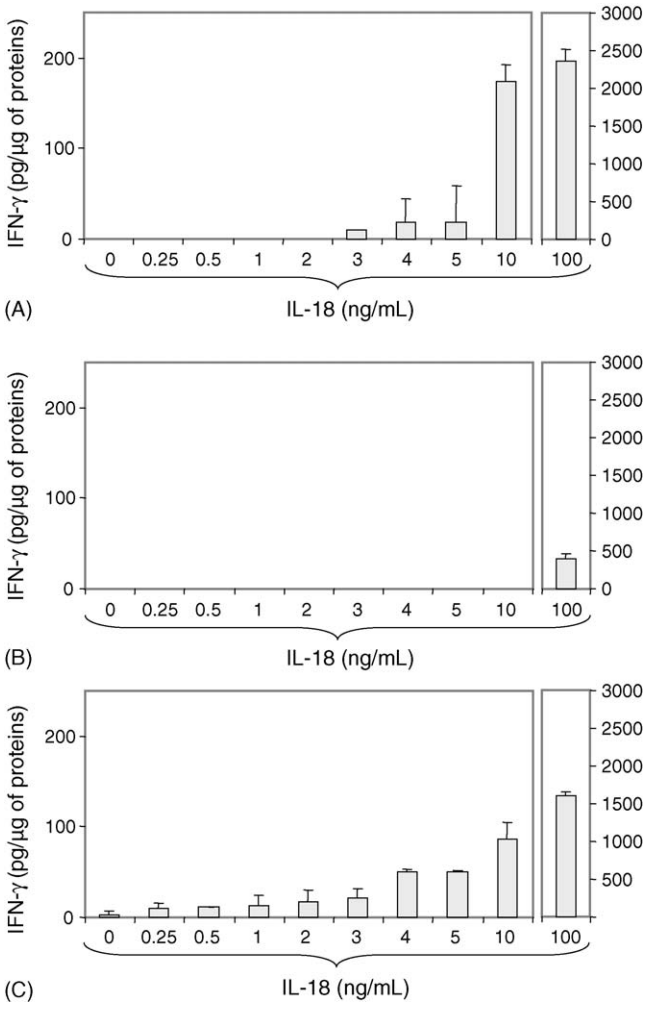

Fig. 3. Consequences of co-lyophilization on IL-18 biological activity. (A) Dose/response histograms representing biological activity of BSA containing IL-18. (B) Dose/response histograms representing biological activity of carrier free IL-18. (C) Dose/response histograms representing biological activity of carrier free IL-18 co-lyophilized with BSA and PEG. Note the restitution of lost activity in (C) by comparison to (B).

These results emphasize that if PEGs of different molecular weights have been demonstrated to stabilize certain proteins, they are also protein cryoprotectants and precipitating/crystallizing agents in aqueous media at high concentrations. Protein stabilization by high-molecular weight PEGs during micronization is likely due to their steric hindrance of protein–protein interactions (for review see, Wang, 1999).

3.4. Consequences to the use of PLGA on IL-18 biological activity

As PLGA microspheres are known to acidify the microenvironment in which they are as far as they degrade, we evaluated the influence of the pH on IL-18 biological activity as well as possible effects of direct interactions between the cytokine and the polymer itself. As shown in Fig. 4A, while after 2 h incubation at pH 2 lead to a slight reduction of biological activity, this effect was not significant and IL-18 biological activity remained

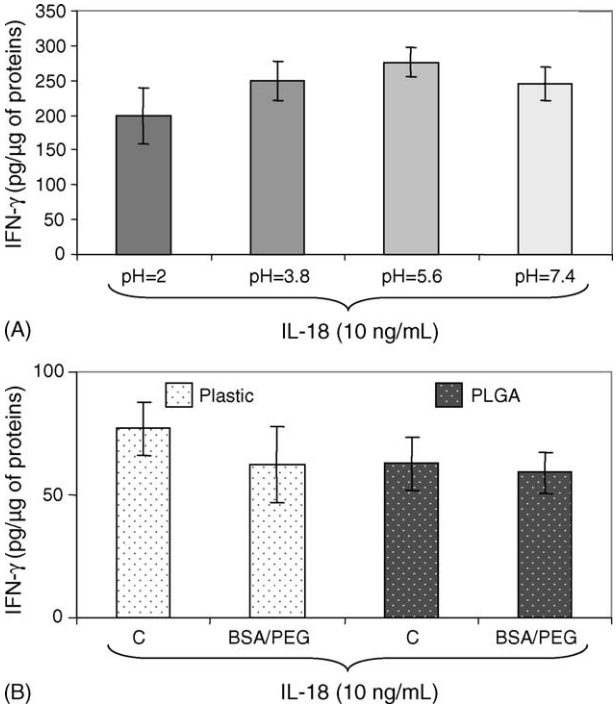

Fig. 4. Consequences to the use of PLGA on IL-18 biological activity. (A) Influence of the pH. (B) Influence of the polymer.

very similar to the one observed at pH between 3.8 and 7.4. Similarly, overnight incubation of IL-18 in MOPS buffer at 37 °C on plastic (polystyrene dishes) or on PLGA (coated dishes) did not result in significant reduction of its biological activity whatever BSA and PEG were present. Such data must be ascribe to the effects of BSA that is thought to scavenge protons during polymer degradation, avoiding any aggregation resulting from acidity (Johansen et al., 1998).

3.5. W/O/W

The microparticles produced by W/O/W emulsion extraction process had a mean volume diameter of 25.1 µm with a standard deviation around the size value of 9.8 µm, the population was monomodal and Gaussian shaped (mode/mean ratio = 1.17) (Fig. 5A and B). The encapsulation efficiency was 85.3%. As shown in Fig. 5E, the release profile of IL-18 was characterized by a first phase of rapid release (60 ng/mg/day) followed by a plateau (4 ng/mg/day). Using the bioassay described in the first part of this paper, we have shown that the biological activity of released IL-18 was maintained during at least 24 days (Fig. 6A).

A previous study performed by our research team was focused on NGF encapsulation by the same encapsulation process and using the same polymer (PLGA 37.5/25) (Pean et al., 1998). The release profile was similar, i.e. 25% burst release after 4 days followed by a plateau. The biological activity was only assessed during the first day of release (bioassay on PC12 cells). Here we have shown that the biological activity was maintained during a much longer period.

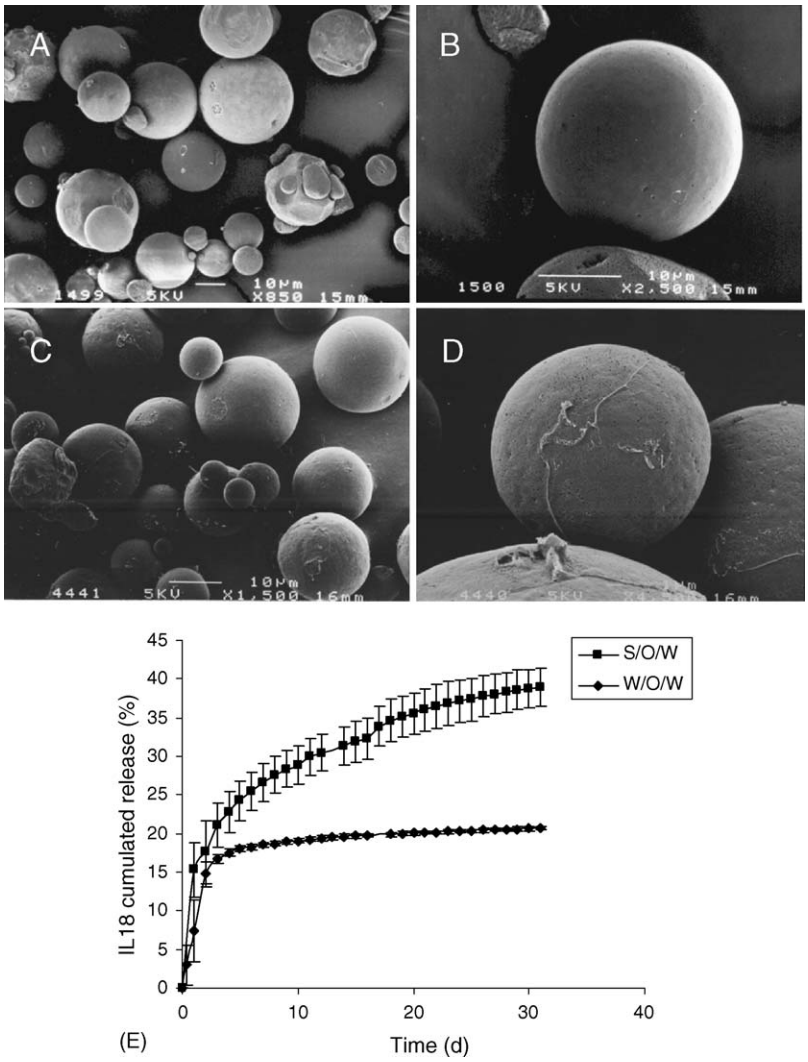

Fig. 5. Characterization of IL-18 microspheres. (A–D) SEM analysis of IL-18 microparticles prepared by W/O/W method (A, ×850; B, ×2500) and S/O/W method (C, ×1500; D, ×4500). (E) In vitro release upon time of total IL-18 from microparticles prepared by W/O/W method (squares) and S/O/W (triangles), calculated as percent of released radioactivity.

The remaining problem was the low pattern of release after the first days. In order to improve the slope of the release, we have tried to change the formulation parameters.

The first idea was to change the concentration of PVA, as it was previously shown that it can have an impact on the release profiled (Soriano et al., 1996). Indeed, the PVA concentration in the aqueous phase has an impact on the microparticles size and on the wetability of the microparticles, both parameters being determinant for the release profile. The concentration of PVA ranged from 2.5 to 10%, the different release profiles are shown in Fig. 7. These results clearly showed that the effect of PVA concentration was only related to the first phase of the release profile and not to the plateau. The same effect of PVA concentration on the release profile have previously been observed with insulin-loaded PLGA microparticles, also prepared by a W/O/W encapsulation process (Soriano et al., 1996). Using experimental design, Erden and Celebi, 1996 have shown that the effect of PVA concentration on release profile is rather complex: this effect of this parameter is dependant from others such as the polymer type, the amount of gelatin in the inner dispersed phase or the drug loading.

To enhance the rate of IL-18 release two different processes were tested. First 10% PLA 2000 oligomers were incorporated in the standard formulation and secondly the entire polymer (PLGA 37.5/25, $\overline{M}_w$ 17,500) was switched to a polymer with a lowest MW and a more equilibrated LA/GA ratio (PLGA 50/50, $\overline{M}_w$ 10,000). The results of modification of the polymer matrix on the release profile are shown in Fig. 8. Both modifications had once again, only an effect on the first phase of the release and not on the plateau. These results are in discrepancy with previous studies performed on small molecules encapsulated in different polymers. We had previously shown that oligomers had an impact not only on the burst effect but also on the rate of release after the burst effect (Geze et al., 1999). But, in these previous studies the drug, which was a small molecule and not a protein, was encapsulated following a S/O/W process, in this case, the

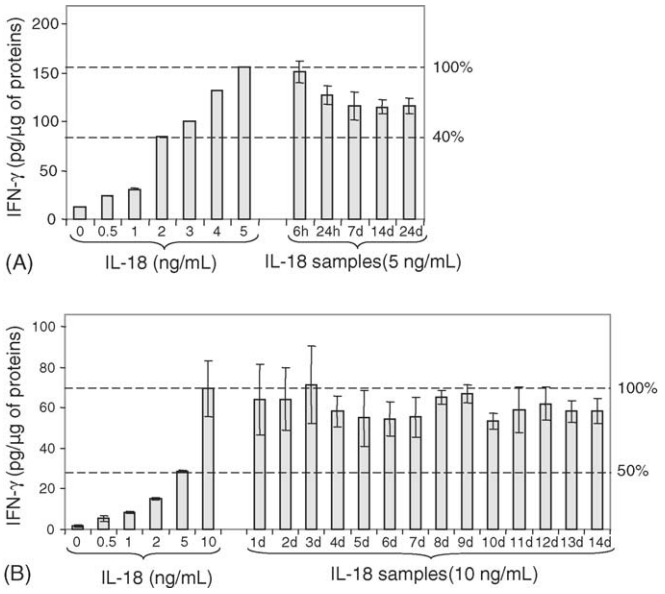

Fig. 6. Determination of IL-18 biological activity after release from PLGA microspheres. Histograms represent one representative experiment on microspheres prepared with W/O/W method (A) and S/O/W method (B). Note the relative biological stability (close to 100% when compared to control) of IL-18 released from microspheres prepared by the S/O/W method (A).

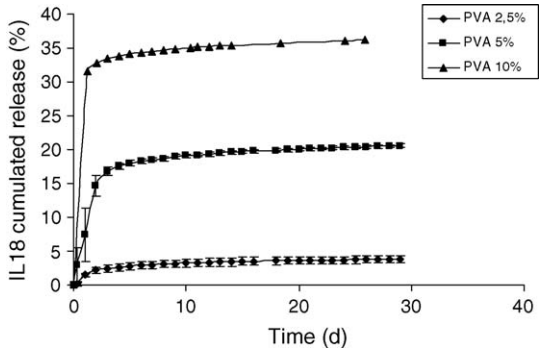

Fig. 7. Impact of the PVA concentration on the release rate of IL-18 from PLGA microspheres calculated as percent of released radioactivity upon time.

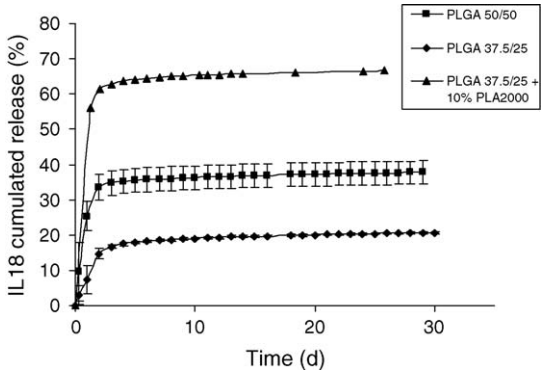

Fig. 8. Impact of the type of polymer and oligomer addition on the release rate of IL-18 from PLGA microspheres calculated as percent of released radioactivity upon time.

interaction between drug crystals and the polymer are greatly reduced, the release is then controlled by the degradation rate of the polymer. Moreover, the interactions of proteins with PLGA (covalent or non covalent aggregation, non specific adsorption) are known to be higher than with small molecules. Indeed, these interactions are often claimed to explain the lack of total release from protein-loaded PLGA microparticles (Aubert-Pouessel et al., 2004). This may explain the discrepancies between these two first studies and the present work. This reflection made us reconsider the entire encapsulation process. In order to be closer to the S/O/W encapsulation of small molecules we tried to encapsulate IL-18 after precipitation by co-lyophilization in PEG. The purpose was to stabilize IL-18 while minimizing its interaction with the polymer to enhanced its release rate.

3.6. S/O/W

The S/O/W process allowed us to produce microparticles with a mean volume diameter of 28.60 μm with a standard deviation around the size value of 11.4 μm (Fig. 5C and D). The encapsulation efficiency was just up to 50% theoretical corresponding to a drug loading of 1.33 μg of IL-18 for 1 mg of microparticles. The release profile was modified as the S/O/W encapsulation method was used (Fig. 5E). The mean release rate between days 4 and 28 was 16.5 ± 8.4 ng/day of IL-18 for 1.5 mg of microparticles. In comparison this rate was only 6.1 ± 4.2 ng/day of IL-18 if W/O/W encapsulation process was used. The burst effect was in both cases around 15%. In S/O/W the interactions between PLGA and proteins are minimized because the protein is in its solid form. The bioassay developed specifically for IL-18 biological activity testing and described previously in this paper showed that the activity of IL-18 remain around 60% during at least 14 days (Fig. 6B).

4. Conclusion

We here formulated PLGA microspheres that could be implanted stereotactically within the CNS and that could release in a controlled manner active IL-18 in accordance with the specifications for in vivo immunotherapeutic applications against gliomas. While both microparticles were able to release IL-18 in a controlled manner during several weeks, the release was faster with S/O/W method than with W/O/W method. Considering daily release together with biological activity, amounts of active IL-18 obtained with S/O/W method, with a moderate burst effect and a subsequent progressive discharge of 16.5 ± 8.4 ng/day during the next 21 days, were sufficient to plan in vivo evaluation to validate the therapeutic strategy. Thus, preclinical safety of recombinant IL-18 has already been documented in mice and monkey (Herzyk et al., 2003). Therefore, together with efforts recently made on cytokine design in order to improve their receptor binding and stability, notably on human IL-18 (Yamamoto et al., 2004), biodegradable recombinant IL-18 releasing microspheres may represent a useful device for the treatment of brain cancers.

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