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International Journal of Pharmaceutics 309 (2006) 1–5
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Development of new polymer-based particulate systems for anti-glioma vaccination

A. Sapin [a], E. Garcion [a], A. Clavreul [a,b], F. Lagarce [a], J.P. Benoit [a], P. Menei [a,b,*]

[a] *Inserm, U646, Université d'Angers, Angers, F-49100, France*
[b] *Département de Neurochirurgie CHU d'Angers, 4 rue Larrey, 49033 Angers Cedex 01, France*

Received 31 January 2005; received in revised form 7 October 2005; accepted 21 October 2005
Available online 28 December 2005

Abstract

Biodegradable and biocompatible microspheres represent a promising alternative to conventional adjuvants for anti-tumour vaccination. Focusing on glioma, we developed two poly(D,L-lactide-co-glycolide) (PLGA)-based particulate systems presenting tumour antigens associated with plasma membranes or with cell lysates. Glioma cell fractions were prepared for adsorption onto poly-D-lysine (PDL)-coated PLGA microspheres formulated using a double-emulsion procedure. Adsorption was followed by ^{125}I-radiolabelling, Western blot and confocal laser scanning microscopy. Only a panel (34%) of the proteins isolated from both cell fractions adsorbed onto PDL-coated PLGA microspheres. The integrity of the epitopes after loading was preserved, as shown by identification of plasma membrane and cytoplasmic markers. Finally, one of the major potential advantages of those particulate systems resides in the fact they not only serve as injectable adjuvant matrices presenting tumour antigens to antigen presenting cells, but also as potential reservoirs for controlled delivery of active immunostimulant molecules.

Keywords: Antigens; Adjuvant; Anti-tumour vaccination; Glioma; Particulate systems; PLGA microspheres

1. Introduction

Anti-tumour vaccination is a promising area of research but identification of specific tumour associated antigens is still more a wish than a reality in many cancers. Tumour cell extracts containing a panel of tumour antigens are often used for cancer vaccination. The immunogenic potential of these tumour cell extracts may vary markedly so there is a need for the development of improved vaccine adjuvants.

Biodegradable poly(D,L-lactide-co-glycolide) (PLGA) microspheres particles can function as an adjuvant to stimulate immune responses (Johansen et al., 2000; Matzelle and Babensee, 2004). PLGA microspheres present the great advantage of being well characterized biocompatible devices (Athanasiou et al., 1996; Menei et al., 1993; Shive and Anderson, 1997), approved for clinical applications (Menei et al., 1999), allowing sustained drug release functions (for review, see Benoit et al., 2000). Such particles carrying antigens can operate as artificial surface presenting antigens for lymphocyte activation (Mescher, 1992; Curtsinger et al., 1997; Kazzaz et al., 2000). Alternatively, they can be taken up by antigen presenting cells (APCs) via the phagocytic route, leading to the presentation of a pattern of antigens to specific immunological effective cells (Scheicher et al., 1995; Audran et al., 2003; Thiele et al., 2003; Elamanchili et al., 2004; Waeckerle-Men et al., 2004).

Focusing on the potential for glioma vaccination, we have developed two PLGA-based particulate adjuvant systems presenting tumour antigens.

2. Formulation of PLGA microspheres

PLGA microspheres were prepared using a double-emulsion solvent extraction–evaporation process previously described (Aubert-Pouessel et al., 2004). In brief, a 0.12 mL internal aqueous phase (1.6 mM citrate buffer containing 5 mg human serum albumin) was mixed with 0.18 mL PEG 400 and emulsified in an organic solution (3 mL methylene chloride and 1 mL

* Corresponding author. Tel.: +33 2 41 73 58 55; fax: +33 2 41 73 58 53.
E-mail address: phmenei@chu-angers.fr (P. Menei).

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doi:10.1016/j.ijpharm.2005.10.046

acetone) containing 100 mg PLGA 37.5/25, M_w = 30,000 Da (Phusis, St. Ismier, France). Emulsification was performed using sonication. The resulting emulsion was subsequently poured into an external aqueous solution of polyvinyl alcohol (60 mL, 5%) containing NaCl (10%) and mechanically stirred at 1100 rpm. The resulting w/o/w emulsion was poured into a large volume of water and stirred for 40 min to complete the extraction of the organic solvent by evaporation. Finally, the microspheres were filtered on a 0.45 μm filter, washed and freeze-dried. The volumetric mean particle size was measured using a Coulter multisizer® (Coultronics, Margency, France).

One restrictive condition for efficient presentation of tumour antigens to lymphocytes concerns their uptake by APCs, and phagocytosis of subcutaneously injected particulate systems by APCs is size dependent (Men et al., 1999; Walter et al., 2001; Waeckerle-Men et al., 2004). While it can still be a matter of debate, macrophages and dendritic cells are able to optimally phagocyte particles of up to 30 and 10 μm in diameter, respectively. For this purpose, microspheres were formulated with a mean diameter of 12 ± 4.6 μm (Fig. 1). This size range would support not only uptake of antigen-loaded microparticles by dendritic cells and macrophages but also further entrapment and size dependent long-term release of therapeutic molecules (Panyam et al., 2003; Siepmann et al., 2004).

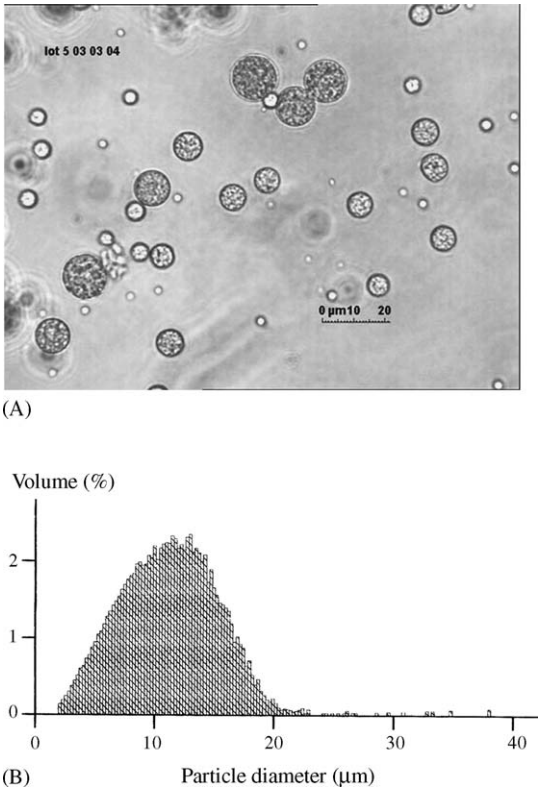

Fig. 1. Characterisation of PLGA microspheres prepared using a double emulsion-solvent evaporation process. (A) Microscopic analysis shows the absence of fragments but a degree of polydispersity and (B) mean particle size determination shows the mean volume diameter to be 12 μm.

3. Adsorption of glioma-antigen-containing cell fractions onto PLGA microspheres and protein stability

Cell lysates and plasma membranes have already been used in cancer vaccination (Lemonnier et al., 1978; Heike et al., 1994; Mescher and Savelieva, 1997; Friedl et al., 2000; Herr et al., 2000; Wang et al., 2000; Schulke et al., 2003). Because of that, we adsorbed these two cell fractions onto microspheres. Plasma membranes and cell lysates, containing a panel of potential tumour antigens derived from rat 9L gliosarcoma cells (9L, LGC, Molsheim, France), were prepared. Plasma membranes were obtained after 2×10^8 cell lysis by hypotonic shock in 42 mM KCL, 10 mM Hepes, pH 7.4, 5 mM $MgCl_2$ and 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF) for 15 min at 4 °C as previously described (Jin et al., 1998). Plasma membranes and intracellular membranes were fractionated by aqueous two-phase partition with a dextran-polyethylene glycol (PEG 8000) biphase as described elsewhere (Heike et al., 1994). Membranes were resuspended in the PEG phase and were underlaid with the dextran phase. The two phases were mixed and centrifuged (3000 *g* for 15 min). The material at the interphase, which was enriched in plasma membranes, was collected and washed twice in sucrose 0.25 M, Tris–HCl 10 mM, pH 7.4 by ultracentrifugation. Enriched plasma membrane material was finally frozen at −80 °C.

The cell lysates were obtained by lysis of 2×10^7 cells following six to eight freeze cycles (in liquid nitrogen) and thaw cycles (37 °C water bath) (Bachleitner-Hofmann et al., 2002).

Using the extraction buffer Tris buffer saline (TBS), 1% of non-ionic detergent Nonidet® P40 (NP40; Calbiochem, San Diego, CA, USA), 1% PMSF and proteases inhibitor cocktail (Calbiochem), the protein content of plasma membranes and cell lysates were determined by a BioRad protein assay kit (Bio-Rad, Marnes-la-Coquette, France). Only 1125 ± 408 μg plasma membrane proteins were isolated from 2×10^8 9L cells (mean from 9 samples), while 5012 ± 1700 μg cell lysate proteins were obtained from 28×10^6 9L cells (mean of 10 samples). Such a difference, that represents a protein yield 32-fold greater in the case of cell lysates, points out an important feature for clinical perspectives since frequent difficulties arise to obtain sufficient numbers of tumour cells in culture.

Microspheres were mixed and maintained in rotation for 15–18 h at 4 °C with plasma membranes or cell lysates previously sonicated. In order to optimise adsorption of both cell fractions containing proteins on PLGA microspheres, various coating molecules (poly-D-lysine (PDL), polyethyleneimine, gelatine and polyornithine) were tested. Our data (not shown) demonstrating a maximal adsorption with PDL, combined with the fact it is pharmaceutically accepted, directed our choice for this coating molecule. In order to further determine the adsorbed protein ratios and to attempt to reach the maximum amount of proteins that can be loaded onto PDL-coated PLGA microspheres, a radiolabelling assay was used. Increasing numbers of microspheres were mixed with 10 μg protein-equivalent cell fractions, previously labelled with the ^{125}I-Bolton Hunter reagent (Perkin Elmer Life and Analytical Sciences, Courtaboeuf, France). In both cases (plasma membranes and

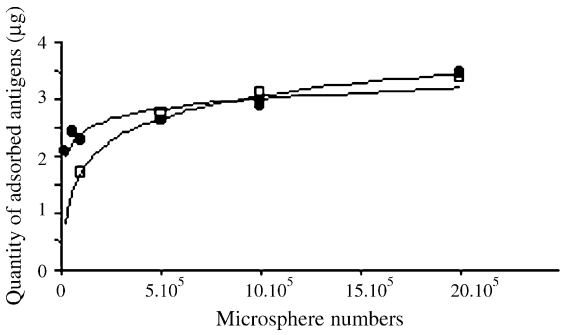

Fig. 2. ^{125}I-Bolton Hunter analysis of the variation of protein adsorption yield related to the protein/microsphere ratio. Adsorption of cell lysate proteins (open square) and plasma membrane proteins (closed circle) was followed by a radiollabelling assay. The quantity of proteins was fixed at 10 μg and mixed with various amounts of microspheres. Adsorption reached a maximum of about 3.4 μg when 10 μg proteins were incubated with 2×10^6 microspheres.

cell lysates) the amounts of proteins that can be adsorbed onto PDL-coated PLGA microspheres reach a plateau (Fig. 2). As microspheres, used in increasing numbers in this assay, are unlikely to represent a saturable system for adsorption of plasma membrane or cell lysate preparations, it is hypothesized that the characteristics of proteins or related organelle compartments that can be loaded onto them constitute a limiting factor. However, despite the difference of material nature between plasma membranes and cell lysates the same maximum protein adsorption yield was obtained (34.7% and 33.9%, respectively). Hence, while it is possible to reach higher yields for single protein adsorption onto PLGA microspheres, as exemplified by the 75% loading obtained with p55gag (Kazzaz et al., 2000), it remains challenging to find a general coating procedure that will work for distinct biological components which behave in different ways.

While the quantity of antigen loading must be a crucial parameter to activate efficient immunological responses, it is also undeniable that the integrity of proteins within new adjuvant systems is key. As shown in Fig. 3A and B, by using Western Blotting under denaturing and reducing conditions, proteins currently found, respectively, in cell lysates (Fas-L and Actin) and in plasma membranes (ICAM-1, Fas-L, 5′-nucleotidase) were fully recognised as a single band at their precise molecular weight during the whole formulation procedure. In addition, immunofluorescence together with confocal laser scanning microscopy (Zeiss LSM 510 microscope (Carl Zeiss SAS, Le Pecq, France) at ×20 and ×40 magnifications) revealed that cell fraction-derived epitopes are detected onto the surface of the newly developed carrier systems as exempli-

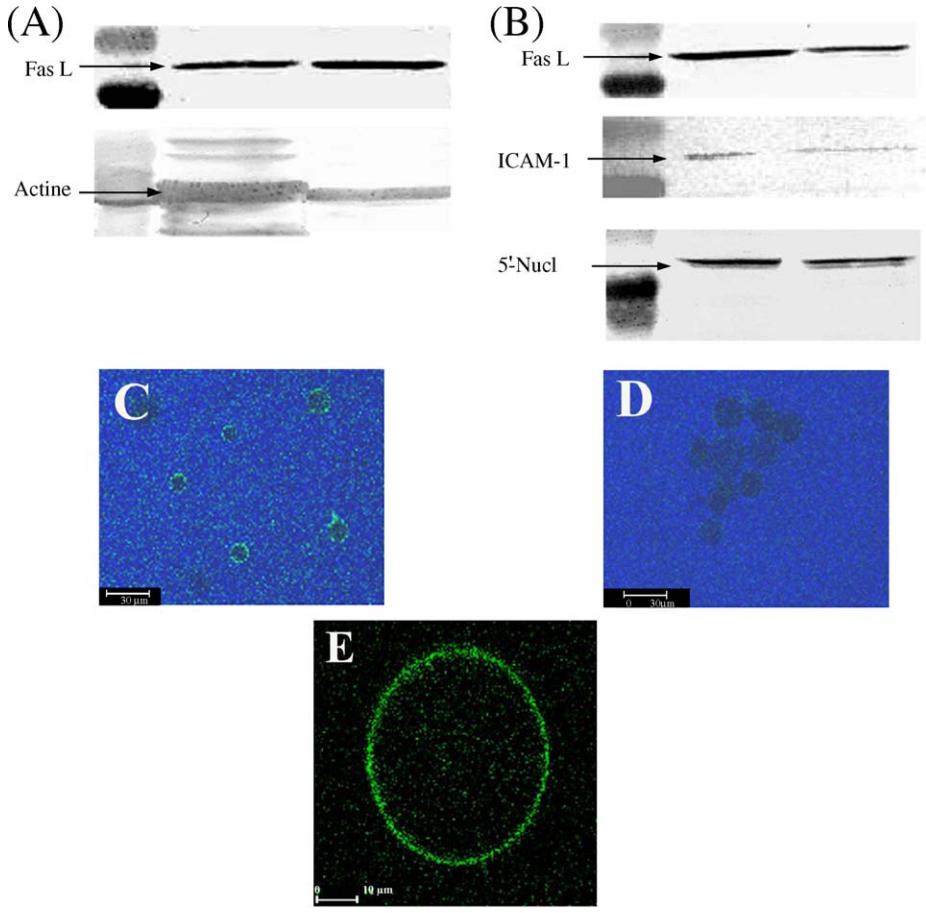

Fig. 3. Verification of the integrity of some marker proteins. (A) Western blot analysis of cell lysate antigens (Fas-L and Actin) and (B) plasma membrane antigens (Fas-L, ICAM-1, 5′-nucleotidase). Protein detection before the adsorption process (lane a) and after the adsorption onto microspheres (lane b). (C and E) Confocal laser scanning microscopy evidenced adsorption of ICAM-1 (marker of plasma membranes) at the surface of PLGA microspheres. (D) No staining was observed in the absence of plasma membranes.

fied by the expression of ICAM-1 on plasma membrane-loaded microspheres (Fig. 3C–E). Thus, PLGA microsphere-based systems presenting glioma antigens appeared as round structures with green fluorescence on their surface (Fig. 3C and E). No staining was observed when microspheres were treated alone (Fig. 3D). All those results support preservation of the integrity of the adsorbed antigens.

4. Conclusion: the double objective device

One of the major intentions for using the developed particulate systems resides not only in their ability to provide an interesting support for tumour–antigen uptake by APCs but also in their potential aptitude to release active cytokines that would regulate APCs activation or/and differentiation (Kupper et al., 1987; Heufler et al., 1988; DiPersio, 1990; Okamoto et al., 1999). Thus, a double emulsion process that we have applied to entrap and release several molecules of interest (Menei et al., 2000; Pean et al., 2000; Aubert-Pouessel et al., 2004; Jollivet et al., 2004) was used for the PLGA microsphere formulation here. As a matter of fact active granulocyte macrophage-cell stimulating factor (GM-CSF) has been loaded into PLGA microspheres by others (Pettit et al., 1997; Mandal et al., 2004) and similar polymer-based formulations releasing cytokines already showed their efficiency in various tumour models (Hill et al., 2002; Ma et al., 2003; Sabel et al., 2004). Hence, PLGA microspheres loaded with interleukin-18 (Golab, 2000) and conjugated with glioma extracts are under investigation in our laboratory.

In conclusion, we have developed particulate systems able to present glioma antigens to immune cells and potentially capable to deliver at the same time immunostimulatory molecules. Preclinical evaluation of this system in animal model of glioma is undergoing.

Acknowledgements

We are grateful to Myriam Moreau, Alf Lamprecht and the “Animalerie Hospitalo-Universitaire d’Angers” for technical support. Anne Sapin was supported by the French “Ministère de l’Education et de la Recherche” and Emmanuel Garcion by the European Commission in the context of the fifth PCRDT (QLK3-CT-2001-02226).

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