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International Journal of Pharmaceutics 269 (2004) 259–265
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Note

Immobilisation of GM-CSF onto particulate vaccine carrier systems

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Received 26 April 2003; received in revised form 17 July 2003; accepted 8 September 2003

Abstract

Physical connection of vaccine carriers with immunostimulating cytokines may provide an interesting possibility to enhance the immune response of protective or therapeutic vaccines. As a first evaluation, various aluminium hydroxide adjuvants and poly(D,L-lactide-*co*-glycolide) (PLGA) microparticulates with modified positively and negatively charged surfaces were prepared to adsorb granulocyte-macrophage colony-stimulating factor (GM-CSF) under different pH conditions. Negatively charged surfaces were chosen to resemble physiological binding of GM-CSF to extracellular glycosaminoglycans, while modified positively charged surfaces may enhance GM-CSF adsorption due to electrostatic interaction. Release of GM-CSF was checked in vitro in a simulated interstitial environment. Anionic and cationic surfaces efficiently attracted GM-CSF to the carrier surface independently of the pH, while the composition of the carrier largely influenced the release of GM-CSF over time. Thus, the adsorption of GM-CSF to aluminium hydroxide adjuvants and PLGA microparticulates provides a simple and efficient possibility to physically connect the cytokine with these commonly used and potential vaccine carriers and may enable its localised delivery to the side of action.

Keywords: Microparticulates; GM-CSF; Immobilisation; Adjuvant; Vaccines; Poly(D,L-lactide-*co*-glycolide) (PLGA)

1. Introduction

Microparticulates have been widely employed as vaccine delivery systems. Aluminium hydroxide microparticulates have a long history of safe and effective performance in both human and animal vaccines. It is suggested that a major part of the antigen should be adsorbed to the aluminium hydroxide microparticulates in order to obtain a physical co-localisation of the antigen and the adjuvant after in vivo injection (Chang et al., 2001; Shirodkar et al., 1990). In addition to aluminium hydroxide vaccine carriers, biodegradable polymeric poly(D,L-lactide-*co*-glycolide) (PLGA) and poly(D,L-lactide) (PLA) microparticulates have a proven track record for safe drug delivery and have been demonstrated to induce efficient immune responses in mice as well as in larger animals, such as monkeys (Johansen et al., 2000; Kazzaz et al., 2000; Singh et al., 2001; Venkataprasad et al., 1999).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a growth and differentiation factor for hematopoietic progenitor cells (Gasson, 1991). Continuous in vivo infusion of GM-CSF increased the number of dendritic cells (Basak et al., 2002), and enhanced immune responses were obtained by administration of GM-CSF (Morrissey et al., 1987; Wang

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doi:10.1016/j.ijpharm.2003.09.023

et al., 2002; Harrison et al., 2002; Kumar et al., 2002; Ou-Yang et al., 2002; Sun et al., 2002). GM-CSF is normally absent from the circulation and is mostly a local factor which is in close proximity to the target cells. Thus, localised delivery is preferred (Chen et al., 2002; Yei et al., 2002) and immune responses may be enhanced by physical connection and co-delivery with the vaccine carrier system. In this sense, controlled release of encapsulated GM-CSF from PLGA microspheres elicited enhanced recruitment of neutrophils, macrophages, dendritic cells and monocytes in a non-human primate model as compared to empty microspheres (Gombotz et al., 1995; Pettit et al., 1997). GM-CSF is recommended to be administered over a period of 1 day to 1 week in order to obtain the optimal effect on white blood cells (Gombotz et al., 1995; Pettit et al., 1997).

The objective of this study was to investigate whether simple adsorption of GM-CSF to the surface of microparticulates is feasible to allow physical connection of the cytokine to the vaccine carrier and, thus, potentially enable localised delivery to the side of action. Adsorption of GM-CSF to the surface of various aluminium hydroxide adjuvant and PLGA microparticulate formulations displaying either positive or negative surface charges was tested under two different pH conditions. Release of GM-CSF was checked in vitro in a simulated interstitial environment.

2. Materials and methods

2.1. Materials

The aluminium containing adjuvant (Rehydragel HPA, Reheis, NJ, USA) was kindly provided by Schweizerhall Chemie AG, Basel, Switzerland. Poly(D,L-lactide-*co*-glycolic acid) (PLGA) type polymer (Resomer RG502H, ratio of lactide:glycolide 50:50, MW 14,000, uncapped end groups) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Polyethyleneimine (PEI) MW 600,000–1,000,000 was from Fluka (Buchs, Switzerland) and poly-L-lysine (PLL), MW 20,000 was purchased from Sigma. All other chemicals used were of analytical grade unless otherwise specified and obtained by Sigma (Buchs, Switzerland).

2.2. Aluminium-containing microparticulates

Aluminium-containing microparticulates displaying a shifted isoelectric point were prepared as described elsewhere (Chang et al., 1997). All resulting adjuvant suspensions were adjusted to 1 mg Al/ml.

2.3. PLGA microparticulates

PLGA microparticulates were prepared by spray drying as previously described (Walter et al., 1999; Walter and Merkle, 2002). Cationic PEI–PLGA microparticulates (20%, w/w PEI nominal loading) were prepared by adding PEI dissolved in 0.2 M phosphate buffer, pH 7.4 immediately before spray-drying (Walter and Merkle, 2002). Cationic PLL–PLGA microparticulates were prepared by coating of plain PLGA microparticles with PLL according to Faraasen et al. (2003). The particulate size distribution was measured by laser light scattering (Mastersizer X, Malvern Instruments Ltd., Worcestershire, UK; equipped with a 45 mm lens) and revealed particles in the range of 1–10 μm as previously described in detail (Walter et al., 1999, 2001).

2.4. Adsorption of GM-CSF to microparticulates

PLGA microparticulates were dispersed in either 50 mM Tris buffer, pH 7.4 or 50 mM Na acetate buffer, pH 5.0 at a concentration of 1 mg/ml. Suspensions of Adjuvants A, B and F (1 ml) were washed once with water prior to use. GM-CSF (R&D Systems Europe, UK) dissolved in water was added (10 ng/mg particulates) and incubated under gentle end to end mixing for 15 min at room temperature. The microparticulates were centrifuged (5 min, 5000 rpm, Eppendorf, Centrifuge 5417R) and 50 μl of the supernatant was diluted with ELISA reagent diluent (1% bovine serum albumin in PBS, pH 7.4) for further analysis by ELISA (R&D Systems Europe, UK). The GM-CSF-loaded microparticulates were washed once with corresponding buffer to remove unbound GM-CSF.

2.5. Release of GM-CSF from PLGA microparticulates and aluminium hydroxide adjuvant in simulated interstitial fluid

GM-CSF-loaded microparticulates (1 mg/ml) were incubated in RPMI 1640 cell culture medium (Life

Technologies AG, Basel, Switzerland) supplemented with 5% (pooled) human serum (Blood Bank Zurich, Switzerland) at 37 °C. Samples were withdrawn at various time point, centrifuged (5 min, 5000 rpm, Eppendorf, Centrifuge 5417R) and the supernatants were analysed for GM-CSF by ELISA (R&D Systems Europe, UK).

3. Results and discussion

GM-CSF is a member of the heparin-binding family and binds to highly anionic sulfate groups of glycosaminoglycans components present in the extracellular matrix due to its positively charged histidine residues (Modrowski et al., 1998; Bernfield et al., 1992; Wettreich et al., 1999). The binding to glycosaminoglycans has been suggested to form a reservoir for GM-CSF and to even promote the biological activity of GM-CSF (Modrowski et al., 1998).

Comparatively, the degree of protein adsorption to aluminium hydroxide adjuvant is influenced by electrostatic forces and involve the isoelectric point (IEP) of the protein and the surface charge of the adjuvant (Chang et al., 1997, 2001). We modified the surface charge characteristics of aluminium hydroxide adjuvant by pre-treatment with phosphate anions in order to obtain positively and negatively charged particulates as characterised by their IEP (11.0–11.3 for Adjuvants A and B and 4.6 for Adjuvant F) according to Chang et al. (1997). Thus, binding to anionic adjuvant may simulate the physiological binding of GM-CSF to anionic glycosaminoglycans as described by others (Modrowski et al., 1998; Wettreich et al., 1999).

A nominal amount of GM-CSF was adsorbed to the various microparticulates which refers to 10 ng per ml of incubation medium. This amount has been demonstrated to be sufficient to stimulate the differentiation of 200,000 monocytes into dendritic cells in vitro (Sallusto et al., 1995; Thiele et al., 2001). In vivo experiments revealed that a tumor vaccine combined with a 1 ng per day infusion of GM-CSF was less effective than a tumor vaccine combined with a 10 ng per day infusion in mice (Chen et al., 2002). We employed physiological (pH 7.4) and low pH (5.0), since the two histidine residues of the GM-CSF molecule provide a positive charge at low pH, thus enhancing binding to anionic surfaces.

Interestingly, there was no noticeable difference in the adsorption of GM-CSF at physiological (pH 7.4) and low (pH 5.0) pH for both the anionic (Adjuvant F) and the cationic (Adjuvants A and B) adjuvants (Table 1). Although lower compared to cationic adjuvant, adsorption of GM-CSF to anionic adjuvant was nonetheless relatively efficient (Table 1). Wettreich et al. (1999) proposed that in an acidic environment and similarly, at neutral pH but in the presence of vesicles containing acidic phospholipids, GM-CSF undergoes conformational transition that enables it to interact with anionic glycosaminoglycans. As suggested for the acidic phospholipids, an acidic microenvironment in close proximity of anionic aluminium hydroxide adjuvant microparticulates may account for the binding of GM-CSF even at neutral solution pH.

An alternative explanation might be that the adsorption characteristic of GM-CSF is in accordance with the findings by Chang et al. (1997) for negatively charged ovalbumin (IEP = 4.6). Ovalbumin was adsorbed by all of the aluminium hydroxide adjuvants (IEP = 4.6–11.3) at pH 7.4, although the adsorptive capacity was greatest with positively charged adjuvants. The adsorption profile of structurally flexible proteins, such as ovalbumin may exhibit a more complex behaviour and is explained by other attractive forces between the anionic protein and the anionic adjuvant, such as van der Waals forces, hydrogen bonding, or hydrophobic interactions (Chang et al., 1997).

Proteins adsorbed to aluminium hydroxide adjuvant are released into the interstitial fluid due to the presence of phosphate anions, citrate anions and interstitial proteins (Chang et al., 2001). The release of GM-CSF from the anionic and cationic aluminium hydroxide adjuvants was investigated in a simulated interstitial environment. We employed cell growth medium containing phosphate anions, supplemented with a low amount of human serum. Human serum was added to avoid adsorption of GM-CSF to the incubation container wall.

There was a slow release of GM-CSF over time for all aluminium hydroxide formulations with release rates between 2.5 and 7.5% of the initial loading at each time point (Fig. 1). Soluble GM-CSF was stable upon incubation under the applied conditions over the period of at least 1 week (data not shown). After 6 days of incubation, about 20–30% of the adsorbed GM-CSF was released in the incubation medium (Table 1). We

Table 1
Loading of GM-CSF onto aluminium hydroxide adjuvants or PLGA microparticulates and amount released over time

No.	Formulation[a]	Loading (ng/ml adjuvant suspension)[b]	Total amount released after 6 days (ng)[c]
1	Adjuvant B loaded at pH 5.0	8.9 (0.15)	2.0 (0.1)
2	Adjuvant A loaded at pH 7.4	9.9 (0.03)	2.1 (0.2)
3	Adjuvant F loaded at pH 5.0	6.9 (0.39)	2.3 (0.1)
4	Adjuvant F loaded at pH 7.4	7.3 (0.02)	2.4 (0.1)
5	PLL–PLGA loaded at pH 5.0	9.7 (0.1)	6.8 (0.3)
6	PLL–PLGA loaded at pH 7.4	9.8 (0.1)	7.8 (0.5)
7	PEI–PLGA loaded at pH 5.0	9.9 (0.1)	12.7 (1.6)
8	PEI–PLGA loaded at pH 7.4	9.9 (0.1)	11.6 (1.6)
9	PLGA loaded at pH 5.0	9.7 (0.1)	12.5 (1.6)

[a] Aluminium hydroxide adjuvants display various isolectric points (Adjuvant A: 11.3, Adjuvant B: 11.0, Adjuvant F: 4.6). PLGA microparticulates contain either a negatively (PLGA) or positively (PLL–PLGA, PEI–PLGA) charged surface.
[b] Nominal loading was 10 ng/ml aluminium hydroxide adjuvant suspension or 10 ng/mg PLGA particulates, respectively. Mean of $n = 3$ with standard deviation given in parenthesis. Values exceeding the nominal loading may be due to variations obtained with the ELISA assay.
[c] Mean of $n = 3$ with squared sum of standard deviations given in parenthesis.

account the desorption of GM-CSF mainly to the dissolution of the aluminium hydroxide adjuvant which has been demonstrated by others in vitro and in vivo in earlier studies (Flarend et al., 1997; Seeber et al., 1991). Organic acids of the interstitial fluid are capable of chelating aluminium and are therefore able to dissolve the aluminium hydroxide adjuvant (Flarend et al., 1997; Seeber et al., 1991). This may explain the uniform release of GM-CSF which was independent on the type of aluminium hydroxide adjuvant and the sampling period, but occurred to a similar extent after each exchange of the incubation medium (Fig. 1). We found indeed by visual observation, the amount of solid aluminium hydroxide adjuvant to decrease over time upon incubation in simulated interstitial fluid by approximately one-third of the initial amount (data not shown). Desorption of GM-CSF due to replacement by phosphate anions or proteins is suggested to play a minor effect. Even the large excess of these anions in the incubation medium has not led to a more rapid release of the protein. Thus, we suggest that GM-CSF that was not released from the aluminium hydroxide remained bound to its surface and would be released upon further dissolution of the aluminium hydroxide particulates.

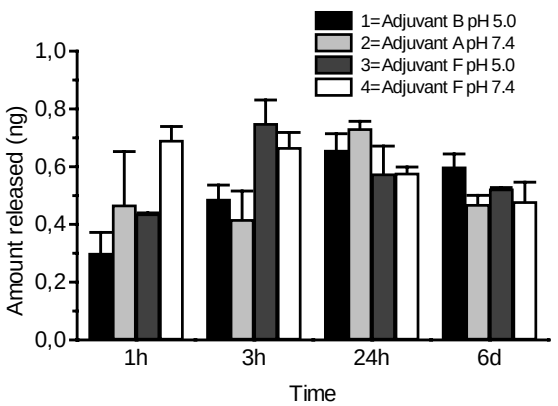

Fig. 1. Release of GM-CSF from aluminium hydroxide adjuvants displaying various isolectric points (Adjuvant A: 11.3, Adjuvant B: 11.0, Adjuvant F: 4.6) at different time points. A total amount 1 mg GM-CSF-loaded aluminium hydroxide adjuvant was added to incubation medium. Error bars represent standard deviations of $n = 3$.

In accordance to aluminium hydroxide adjuvants, we employed positively and negatively charged PLGA microparticulates. Plain PLGA microparticulates prepared by spray-drying were previously reported to display a strongly negative surface charge (Walter and Merkle, 2002). The incorporation of polymeric PEI results in PLGA microparticulates with a positive surface charge (Walter and Merkle, 2002). In addition, the negative surface charge of plain PLGA microparticulates attracts positively charged PLL from the surrounding solution and PLGA microparticulates could be surface-loaded with PLL (Faraasen et al., 2003; Müller et al., 2003). GM-CSF was efficiently adsorbed onto the surface of negatively charged PLGA and positively charged PEI–PLGA and PLL–PLGA microparticulates at neutral (pH 7.4) and at moderately acidic pH (5.0). The lack of any noticeable preference for one

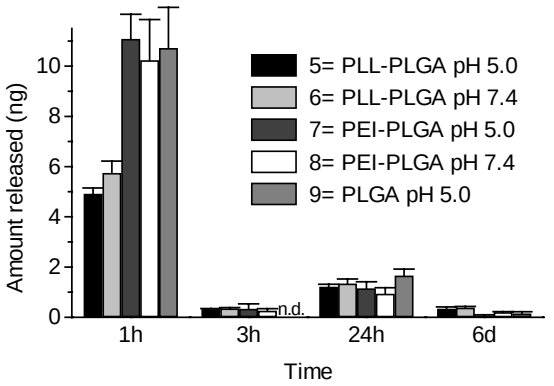

Fig. 2. Release of GM-CSF from plain and modified PLGA microparticulates displaying either a negatively (PLGA) or positively (PLL–PLGA, PEI–PLGA) charged surface. A total amount 1 mg GM-CSF-loaded PLGA microparticulates was added to incubation medium. Error bars represent standard deviations of $n = 3$.

of the PLGA microparticulate formulations as shown in Table 1 may be explained by the generally hydrophobic character of the PLGA microparticulate surfaces (Walter et al., 2001; Walter and Merkle, 2002). In that sense, the attraction of GM-CSF to the PLGA surfaces is in line with the pronounced adsorption of serum proteins, such as albumin, to various PLGA microparticulates (Luck et al., 1998).

Immediate release of large quantities of GM-CSF was found from all PLGA microparticulate surfaces in combination with a delayed release of relatively small amounts over a time span of 6 days (Fig. 2). Interestingly, cationic PLL-modified PLGA microparticulates appeared to retain approximately half of the total amount of GM-CSF on the surface, whereas cationic PEI-modified PLGA microparticulates displayed almost complete release of most of the GM-CSF similarly to plain anionic PLGA microparticulates (Fig. 2). Thus, not only the surface charge, but also the specific character of the surface modification influenced the release characteristics of the adsorbed GM-CSF.

The substantial release of GM-CSF from PLGA microparticulates is in noticeable contrast to the release from aluminium hydroxide adjuvant (Fig. 1). The comparatively immediate desorption from PLGA microparticulates may be mainly explained by the replacement of GM-CSF by the excess of proteins in the incubation medium and is in accordance with its suggested attraction to the microparticulate surface by hydrophobic interaction. PLL-modification of the surface may contribute to a tighter binding due to additional electrostatic interactions.

Physical deposition and localised delivery of cytokines and growth factors has been obtained by encapsulation into microparticulates (Meinel et al., 2001; Pettit et al., 1997) or by incorporation into protein or polysaccharide matrices (Brown et al., 2001; Hubell et al., 2000; Wee and Gombotz, 1998). From a manufacturing point of view, spontaneous adsorption of the active compound to the carrier under appropriate conditions appears to be superior, since the manufacturing process of the carrier would be separate from adsorptive loading and not affect the integrity of the active compound.

In summary, the adsorption of GM-CSF to aluminium hydroxide adjuvants and PLGA microparticulates provides a simple and efficient possibility to physically connect the cytokine with these commonly used or potential vaccine carriers and thus, provides localised delivery to the site of action. Anionic and cationic surfaces have been demonstrated to efficiently attract GM-CSF to the carrier surface, while the composition of the carrier influences the release of GM-CSF over time. Whether the delayed release from a reservoir such as from the vaccine carrier is advantageous for the therapeutic efficiency of GM-CSF needs to be evaluated. In addition, the biological effect of GM-CSF may be enhanced while presented in association to the carrier surface comparable to binding to the extracellular matrix.

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