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Potential of Albumin Nanoparticles as Carriers for Interferon Gamma

Sara Segura, Socorro Espuelas, María Jesús Renedo, and Juan Manuel Irache
Centro Galénico, Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Navarra, Pamplona, Spain

ABSTRACT Although interferon gamma (IFN-γ has been extensively studied as a potent activator for macrophages and as a promising adjuvant in vaccines, its rapid biodegradation and clearance have severely limited its clinical efficacy. Our major objective in this work was to develop formulation conditions to get high association of the cytokine to albumin nanoparticles, without leading any conformational changes and subsequent loss of activity. To achieve this objective, two different formulations were prepared by either 1) incubation between the cytokine and the newly prepared nanoparticles (IFN-NPA) or 2) between the protein and IFN-γ prior coacervation (IFN-NPB). Steady-state fluorescence emission spectra revealed that the environment of the tryptophan (Trp) residue was not affected by conditions of mechanical stress required for preparing nanoparticles. A bioassay for antiproliferative activity with Hela cells indicated that the cytokine, after their desorption from the surface of nanoparticles (IFN-NPA), fully retained its activity. It also indicated that the cytokine was principally associated with nanoparticles via electrostatic interactions and confirmed by desorption experiments carried out in media with different pH and ionic strength, with burst effect ranked in the order pH 5>pH 7.4>pH 8.5. Also, the adsorption of IFN-γ onto these carriers was able to improve the priming effects of IFN-γ on the nitric oxide production (NO) by RAW macrophages. On the contrary, when we incubated the cytokine with the albumin solution prior to the desolvation process for preparing nanoparticles (IFN-NPB), we obtained better encapsulation efficiencies (around 100%), but the cytokine was inactive: it was not detected by ELISA or bioassay in Hela cells and unable to stimulate NO production by macrophages.

KEYWORDS Nanoparticles, Albumin, Interferon-gamma, Immunotherapy

INTRODUCTION

Interferon-gamma (IFN-γ) or type II interferon is a homodimeric glycoprotein endogenously produced by T lymphocytes and natural killer (NK) cells. IFN-γ promotes T and B cell proliferation, activation of mononuclear phagocytes and neutrophils, augmentation of NK cell lytic function, expression of MHC I and II, and suppression of IL-4 responses (Hubel et al., 2002). Moreover, this cytokine displays a wide variety of

Address correspondence to Dr. Juan Manuel Irache, Centro Galénico, Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Navarra, Irunlarrea, 1, Ap. 177, 31008 Pamplona, Spain; Fax: +34 948-42-56-49; E-mail: jmirache@unav.es

antiviral, antiproliferative, immunomodulatory, and apoptotic functions (Le Page et al., 2000).

In 1990, IFN-γ (Actinmune®) was approved by the U.S. Food and Drug Admistration (FDA) to reduce the frequency and severity of infections associated with chronic granulomatous disease (CGD), and to delay the progression of malignant osteopetrosis (Younes & Amsden, 2002). In addition, IFN-γ may also have therapeutic potential for other specific immunodeficiency diseases such as hyper-IgE syndrome (Jeppson et al., 1991) and appears to be effective when used in conjunction with conventional antimicrobial chemotherapy in cutaneous and visceral leishmaniasis (Badaro et al., 1990), disseminated atypical mycobacterial infections, (Holland et al., 1994) and lepromatous leprosy (Murray, 1994). Unfortunately, the clinical use of IFN-γ is limited by its rapid blood clearance and systemic toxicity (Gallin et al., 1995). When administered by the intravenous route, IFN-γ shows an elimination half-life of about 25–35 min (Wills, 1990). On the other hand, high IFN-γ concentrations may induce severe side effects including neurological disorders and leukopenia (Younes & Amsden, 2002).

The conjugation of proteins and cytokines with polyethylene glycol has been a strategy successfully applied to protect them from degradation and to increase their blood circulation time (Morpurgo & Veronese, 2004). In fact, pegylated IFN alpha has already been marketed (Pegasys®) and is currently used for the treatment of hepatitis C (Foster, 2004). However, sometimes pegylated proteins are inactive (Morpurgo & Veronese, 2004).

Another appropriate strategy may be the use of drug delivery systems able to both target and control the cytokine release near the macrophages (its main site of action). In this context, different approaches have been proposed including the use of liposomes (Herrmann & Stricker, 1995; Hockertz et al., 1989; Ishihara et al., 1991; Saravolac et al., 1996; van Slooten et al., 2001a), biodegradable microspheres (Cleland & Jones, 1996; Yang & Cleland, 1997), and adenoviral vectors (Lei et al., 2000; Stoeckle et al., 1996). An alternative way to increase the therapeutic efficiency of IFN-γ may be its loading in albumin nanoparticles. Nanoparticles display relatively higher intracellular uptake compared to microparticles and, in general, better controlled-release properties than liposomes. What is more, albumin nanoparticles can incorporate a wide variety of hydrophilic drugs in a relatively nonspecific way including cationic (i.e., gancyclovir; Merodio et al., 2001) and anionic (i.e., antisense oligonucleotides; Arnedo et al., 2002) molecules. In addition, these biodegradable carriers can be easily prepared under soft conditions by coacervation or controlled desolvation processes avoiding the use of organic solvents and minimizing the possibilities for cytokine degradation.

The aim of the present study was to evaluate the capacity of albumin nanoparticles as carriers for IFN-γ. For this purpose, IFN-γ-was either adsorbed or entrapped in albumin nanoparticles. The influence of different parameters on the physico-chemical properties and in vitro release profile of the resulting nanoparticles were studied. In addition, the specific modulation of cell-mediated immunity by IFN-γ-loaded nanoparticles was determined by the activation of macrophages RAW 264.7, measured as production of nitric oxide (NO).

MATERIALS AND METHODS

Materials

Recombinant human and mouse IFN-γ with a specific activity of 1.0×10^7 UI/mg and IFN-γ ELISA kits were obtained from Biosource International (USA). Bovine serum albumin (BSA, fraction V), glutaraldehyde (grade II, 25%), sodium hydroxide, sodium chloride, copper (II) sulfate, bicinchoninic acid solution, lipopolysaccharide (LPS) (isolated from *E. coli* serotype 0111-B4), and MTT reagent were obtained from Sigma Chemical Co. (St. Louis, MO). Ethanol absolute and chlorhydric acid were purchased from Prolabo (Fontenay, France). Potassium dihydrogen phosphate, monoacid sodium phosphate dodecahydrated, citric acid, and sodium nitrite from Merck (Darmstadt, Germany). DMEM medium, FBS, and L-glutamine were obtained from Gibco-BRL (USA).

Preparation of IFN-γ-Loaded Nanoparticles

Bovine serum albumin nanoparticles were prepared by coacervation and chemical cross-linkage with glutaraldehyde as described previously (Arnedo et al., 2002). Depending on the step in which the cytokine

was added, two different formulations were prepared. In the former, IFN-γ was incubated with the albumin nanoparticles (IFN-NPA). In the latter, both the protein and the cytokine were incubated in an aqueous medium prior to the formation of the nanoparticles by coacervation (IFN-NPB).

Briefly, IFN-NPA was obtained by the addition of 1.5 mL of ethanol dropwise (ethanol-to-water ratio 1.5:1 by vol.) to 1 mL of an aqueous solution of bovine serum albumin (2% w/v), adjusted to 5.5 with HCl 0.1 N. Coacervates thus obtained were then hardened with glutaraldehyde previously dissolved in ethanol (1.56 μg/mg bulk albumin) for 2 h at room temperature. After ethanol elimination by evaporation under reduced pressure (Buchi waterbath B-480, Switzerland), nanoparticles were purified by centrifugation at 27,141 *g*. for 30 min (Rotor 3336, Biofuge Heraeus, Hanau, Germany) to eliminate the free albumin and the excess of the cross-linking agent. The supernatants were removed and the empty nanoparticles (NP) resuspended in an aqueous solution containing different amounts of IFN-γ (ranging from 0.05 to 2.5 μg/mg nanoparticle) for incubation for 30 min. Finally, IFN-NPA were separated from the free drug by centrifugation (27,141 *g*/30 min) at 4°C.

For IFN-NPB, a variable amount of the cytokine (ranging from 0.05 to 2.5 μg/mg nanoparticle) was firstly incubated with the albumin aqueous solution (2% w/v; pH 5.5) for 30 min at room temperature. This aqueous phase was then desolvated with ethanol dropwise (ethanol-to-water ratio 3:2 by volume). Coacervates so formed were then hardened with glutaraldehyde previously dissolved in ethanol (1.56 μg/mg bulk albumin) for 2 h at room temperature and the resulting nanoparticles purified by centrifugation as described above.

In all cases, the purified nanoparticles by centrifugation were resuspended and dispersed in 1 mL of water. As control, unloaded nanoparticles (NP) were used.

Physicochemical Characterization of Nanoparticles

The size and zeta potential of loaded and unloaded albumin nanoparticles were determined by photon correlation spectroscopy (PCS) and electrophoretic laser doppler anemometry, respectively, using a Zetamaster analyzer system (Malvern Instruments, UK). The samples were diluted with distilled water and measured at room temperature with a scattering angle of 90°. All measurements were performed in triplicate.

The amount of protein transformed into nanoparticles was determined by a standard BCA protein assay (Smith et al., 1985). The nanoparticulate pellet, obtained after centrifugation, was digested with NaOH 0.1 M under magnetic stirring for 2 h at room temperature. Then, the resulting solutions were analyzed in a spectrophotometer at 562 nm. In all cases, the resulted absorbance was compared with the data obtained after digestion of a control albumin solution. A rectilinear calibration curve from 10 to 100 μg/mL ($r^2>0.999$) was performed using a control albumin solution in NaOH 0.1 M.

Determination of the IFN-γ Loading and Encapsulation Efficiency

IFN-γ analysis was determined by ELISA in the supernatants after purification of nanoparticles by centrifugation. For this purpose, aliquots of the clear supernatants obtained during the purification of the nanoparticles were analyzed for IFN-γ content. The amount of the cytokine was estimated from the difference between the initially added and the recovered amount in the supernatants. The IFN-γ loading was expressed as the ratio between the amount of cytokine in the nanoparticles (expressed in μg) and the albumin nanoparticle yield (expressed in mg).

Steady-State Fluorescence Measurements

In order to study the influence of the preparative process of nanoparticles on the IFN-γ conformation, steady-state fluorescence measurements were performed. For this purpose, the IFN-γ fluorescence emission spectra (λ excitation=295 nm) were recorded from 300 to 450 nm on a LS-50B Luminiscence spectrophotometer (Perkin Elmer, Norwalk, CT). IFN-γ samples in water acidified with HCl 0.1 N at pH 5.5 (containing 10 μg/mL) were treated with ethanol and glutaraldehyde in the same way as described to prepare nanoparticles. Ethanol was eliminated by evaporation and the resulting solution centrifuged at 27,141 *g* for

30 min (see Preparation of IFN-γ-Loaded Nanoparticles). As control, an IFN-γ solution in water was used.

In Vitro Release Studies of the Loaded IFN-γ

Albumin nanoparticles containing 1 μg IFN-γ, were dispersed in 1 mL of release medium in eppendorf tubes. The release media used here were either PBS pH 7.4 (0.05, 0.15, or 0.5 M), citrate buffer 0.05 M (pH 3, pH 5, or pH 7.4), or borate buffer 0.05 M (pH 8.5 or pH 10). Then, eppendorf tubes were placed in a shaking bath at 37±1°C with a constant agitation of 60 strokes/min (Unitronic 320 OR, Selecta, Spain). At predetermined intervals, the samples were centrifuged and the supernatants assessed for IFN-γ content by ELISA, whereas its biological activity was evaluated using a bioassay. Each experiment was performed in triplicate.

In Vitro Determination of the IFN-γ Activity

HeLa 229 cells were maintained in growth on 75 cm^2 dishes in DMEM supplemented with 10% fetal bovine serum and antibiotics. The cells were cultured at 37°C in a water-saturated 5% CO_2, 95% air atmosphere and plated at a density of approximately of 3×10^3 cells/100-μL medium/well in 96-well culture plates. Then, 24 h later, a standard solution of IFN-γ was diluted into the culture medium and added to the cultured cells (from 0.01 to 500 ng/mL) at the same time as aliquots of the release samples. The cells were further grown for 5–6 days. After this time, the MTT reagent was added and absorbances were measured in a microplate ELISA reader after incubation for 4 h. Absorbance is proportionally related to the number of live cells.

Production of NO by RAW 264.7 Macrophages

IFN-γ, free or loaded in albumin nanoparticles, was assessed by measuring the NO produced by RAW 264.7 cells. Macrophages were seeded in 96-well flat-bottom culture plates (1×10^5 cells in 0.1 mL/well) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. After 2 h, nonadherent cells were removed by washing the monolayers with PBS, and 0.1 mL of complete DMEM medium was added. The different IFN-γ formulations, at a cytokine concentration of 20 ng/mL, were dispersed in 0.1 mL complete DMEM medium with or without LPS of *E. coli* 011:B4 (100 ng/mL) and incubated with cells. After 24 h of incubation in a 5% CO_2 incubator at 37°C, the plates were centrifuged (10 min × 250 g) and the supernatants (0.1 mL) collected. NO_2^-, one of the end products of NO synthesis, was measured by mixing 100 μL of cell supernatant with 200 μL of the Griess reagent and determining the absorbance at 540 nm in a microplate ELISA reader (Labsystems iEMS). Sodium nitrite ($NaNO_2$) diluted in culture medium was used to perform the standard curve.

Statistical Analysis

All experiments were repeated at least three times. Results were expressed as means±standard deviation. Uptake data were analyzed by one-way ANOVA with posthoc Tukey's test applied for comparisons of several (≥ 3) group means (SPSS 10, SPSS Inc., Chicago). $p<0.05$ was considered significant.

RESULTS

Influence of the Preparation Method of Albumin Nanoparticles on the Stability of IFN-γ

It has been shown that the fluorescence of the tryptophan residue in IFN-γ is extremely sensitive to processes of dissociation of the active protein dimers into monomers and thus, aggregation (Boteva et al., 1996; van Slooten et al., 2000). The emission spectra after excitation at 295 nm display emission only from Trp and a maximum were observed at 341 nm for both, untreated cytokine and also the IFN-γ that underwent mechanical stress for the preparation of nanoparticles, namely magnetic stirring, organic solvents, processes of evaporation under reduced pressure, and addition of glutaraldehyde (Fig. 1). Interactions between protein and surfaces (as their adsorption onto the surface of liposomes or nanoparticles) by electrostatic or hydrophobic interactions often leads to unfolding of the protein, and thus, to its inactivation. However, it was not possible to accurately determine either the fluorescence spectra of nanoparticle-associated IFN-γ or that of IFN-γ

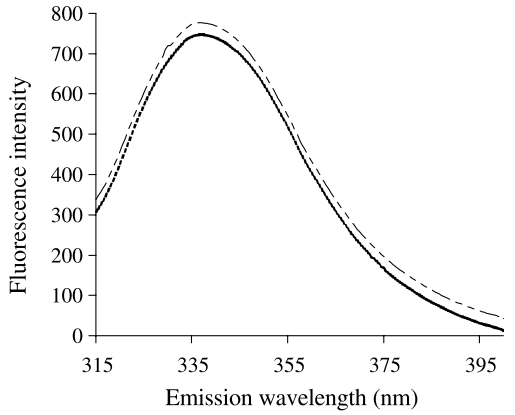

FIGURE 1 **Fluorescence Emission Spectrum of IFN-γ Solution, Excited at 295 nm. Key: (Solid Line) IFN-γ in Water; (Stripped Line) IFN-γ After Exposition to the Nanoparticle Preparation Procedure.**

released from nanoparticles because of a high overlapped signal displayed by the albumin.

Physico-Chemical Optimization

Influence of the Bulk IFN-γ

Figure 2 shows the influence of the IFN-γ concentration on the size and the zeta potential of the resulted nanoparticles.

For both formulations, the size of nanoparticles slightly increased by increasing the IFN-γ-to-albumin ratio, from 300 to around 350 nm. About superficial

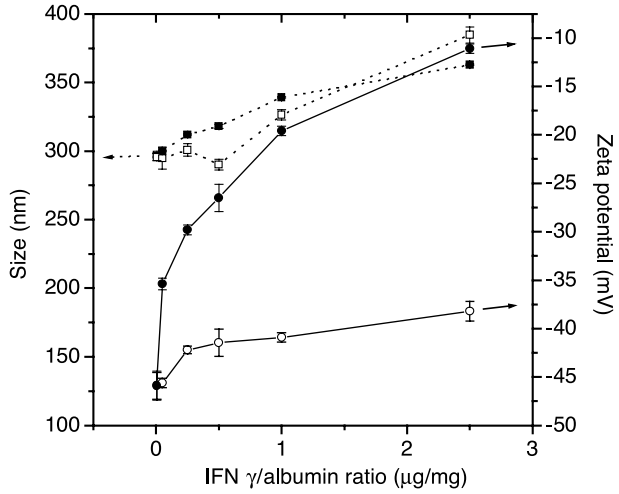

FIGURE 2 **Influence of the IFN-γ/Protein Ratio on the Size (nm) (Stripped Line) and the Zeta Potential (mV) (Solid Line) of the Resulting Nanoparticles. (Open Symbol) IFN-NPA, Nanoparticles Incubated for 2 H with the Cytokine. (Closed Symbol) IFN-NPB, Nanoparticles Prepared after 30 min of Incubation Between the Albumin Aqueous Solution and the Cytokine. Data Express the Mean ± S.D. (n = 3). Nanoparticles were Prepared with a Glutaraldehyde Concentration of 1.56 μg/mg.**

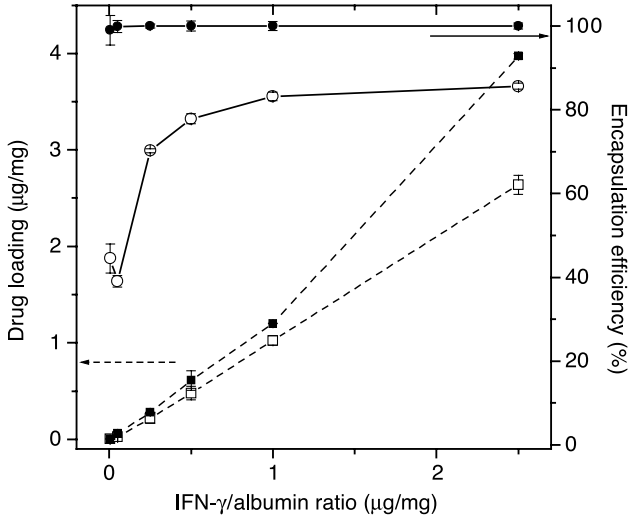

FIGURE 3 **Influence of the IFN-γ-to-Protein Ratio on the Loading (Stripped Line) of the Cytokine in Albumin Nanoparticles and Encapsulation Efficiency (Solid Line). (Open Symbol) IFN-NPA, Nanoparticles Incubated for 2 H with the Cytokine. (Closed Symbol) IFN-NPB, Nanoparticles Prepared after 30 min of Incubation Between the Albumin Aqueous Solution and the Cytokine. Data are Expressed as the Mean ± S.D. (n = 3).**

charge, for IFN-NPA, the negative zeta potential of the unloaded carriers (around −45 mV) dramatically decreased by increasing the amount of the IFN-to-albumin ratio. On the contrary, for IFN-NPB (Fig. 2), little modification on the zeta potential of the albumin carriers was found by increasing the cytokine-to-albumin ratio.

Figure 3 shows the capacity of the two different procedures to incorporate IFN-γ as a function of the cytokine-to-albumin ratio. For IFN-NPA, the IFN-γ loading by adsorption to the surface of albumin nanoparticles increased with the concentration of the cytokine. However, the efficiency of the IFN-γ loading increased up to the level at which the ratio was 0.5 μg/mg. Above this level, the encapsulation efficiency was constant and close to 85%.

For IFN-NPB, the cytokine loading also increased by increasing the cytokine-to-polymer ratio (Fig. 3). However, this loading was always higher than for IFN-NPA. In addition, the entrapment efficiency was calculated to be always close to 100%.

Influence of pH and Ionic Strength on the Adsorption of IFN-γ Onto Albumin Nanoparticles

Cytokines usually have a high cost. Thus, it is particularly appealing to optimize manufacturing conditions to achieve high encapsulation efficiencies

TABLE 1 Influence of the pH and the Ionic Strength on the Zeta Potential and the Capacity of Albumin Nanoparticles to Load IFN-γ

Ionic strength	pH	Zeta potential (mV)	IFN loading (ng/mg BSA)	Encapsulation efficiency (%)
0.05 M	5	−17.6±3.5	21.41±0.22	34.25%
	6	−27.5±1.9	50.34±0.59	80.10%
0.05 M	7.4	−35.1±0.8	53.12±3.34	85.01%
0.15 M		−32.4±1.3	47.09±1.98	75.35%
1.00 M		−36.1±0.8	42.87±0.07	68.60%
0.05 M	8.5	−40.7±1.2	55.66±0.33	89.57%
	10	−53.8±0.9	22.56±2.36	36.09%

Experimental conditions: IFN-γ-to-BSA ratio of 0.05 μg/mg. Data are expressed as the mean±s.d. (n=3).

in the delivery systems. Pursuing this target, we evaluated the influence of both the pH and ionic strength of the incubation medium on the incorporation of IFN-γ in the albumin nanoparticles. Moreover, this experiment can also give us information about the nature of interactions between them, and then, we can also predict if the association can affect the conformation of the protein. The results are summarized in Table 1.

A preferential binding was observed at pH 6–8.5 (Table 1). However, at pH 5 (albumin p*I*, Peters, 1985) and pH 10 (close to cytokine p*I*, Rinderknecht et al., 1984) a certain amount of IFN-γ was still adsorbed to the surface albumin nanoparticles (21.41 and 22.6 ng/mg for pH 5 and pH 10, respectively).

At a fixed pH of 7.4, the adsorption of IFN-γ to unloaded albumin nanoparticles was found to decrease by increasing the ionic strength (Table 1).

Characteristics of IFN-γ-Loaded Albumin Nanoparticles

Table 2 summarizes the main physico-chemical characteristics of the different IFN-γ loaded albumin nanoparticulate formulations, prepared with an IFN-γ-to-albumin ratio of 1 μg/mg.

Under these experimental conditions both types of nanoparticles displayed a size close to 330 nm, significantly higher than for unloaded nanoparticles. On the other hand, the IFN-γ loading was around 1.2 times higher when the cytokine was incubated with the native protein, prior to the formation of nanoparticles, (IFN-NPB) than when it was adsorbed onto the preformed carriers (IFN-NPA). For both procedures, the encapsulation efficiency was high, although the encapsulation process was more efficient than the adsorption procedure.

IFN-γ In Vitro Release from Albumin Nanoparticles

IFN-NPA and IFN-NPB formulations were tested for in vitro release at 37±1°C. The amount of the released protein was analyzed by ELISA and bioassay in Hela cells. Figure 4 displays the plot of the data expressed as the cumulative amount of IFN-γ released from the two albumin nanoparticle formulations as a function of time.

The two formulations displayed different release profiles. The desorption of IFN-γ from nanoparticles showed a biphasic profile, with an important burst effect, in which about 40% of the loaded cytokine was released, followed by a slower release step. Affected by media conditions, the burst effect strongly decreased by increasing pH (from 35% at pH 5 to 8% at pH 8.5, see Table 3) and it was higher at low ionic strength concentrations.

For IFN-NPB, after 100 h only 1% of the released cytokine was detected by ELISA. Neither the ELISA nor the bioassay technique were able to detect the

TABLE 2 Physico-Chemical Characteristics of the IFN-γ-Loaded Nanoparticles

	Size (nm)	Yield (%)	Zeta potential (mV)	Drug loading (μg IFN-γ/mg NP)	Entrapment efficiency (%)
NP	297±3	80.2±1	−46.3±1.1	–	–
IFN-NPA	339±7	80.2±1	−19.6±0.5	1.02±0.30	83.2±0.83
IFN-NPB	326±3	90.3±3	−40.9±0.5	1.20±0.12	99.9±1.41

Experimental conditions: IFN-γ-to-BSA ratio of 1 μg/mg. Data are expressed as the mean±s.d. (n=3). NP: unloaded nanoparticles.

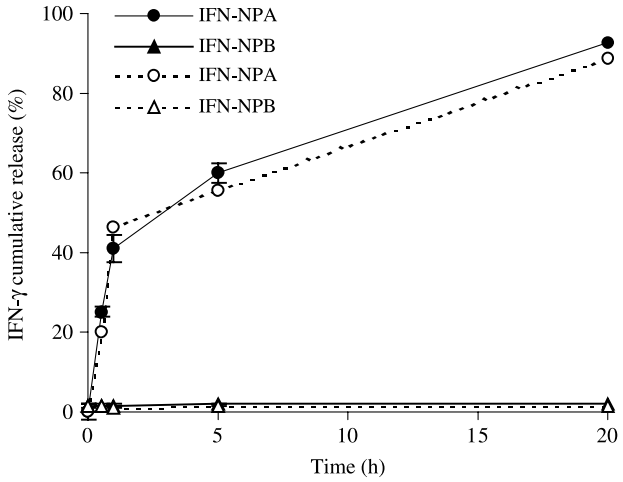

FIGURE 4 **Comparison of the Released Interferon Gamma from Albumin Nanoparticles in PBS (pH 7.4, 0.15M) at 37°C as Measured by ELISA (Open Symbols) and by the Bioassay (Closed Symbols).**

release of the cytokine. Similarly, when IFN-NPB formulations where incubated at different pH and/or ionic strength conditions, no release was observed (data not shown).

Production of NO by RAW 264.7 Macrophages

Finally, we investigated the effect of the loading of IFN-γ in albumin nanoparticles on the nonspecific activation of macrophages. Table 4 summarizes the production of NO by RAW macrophages when treated with the different IFN-γ formulations. Both nanoparticle formulations differed in their ability to stimulate NO production. IFN-NPA induced the production of greater amounts of NO than free IFN-γ (Table 4). The increase was modest but significant ($p<0.05$). On the contrary, when the cytokine was loaded into albumin nanoparticles (IFN-NPB), its activity was inhibited, in a good relationship with no detection of active cytokine in the release studies for this formulation. In addition, control NP was also unable to induce the NO production by RAW macrophages.

TABLE 3 **Influence of the pH and Ionic Strength on the Burst Effect of IFN-γ from Albumin Nanoparticles (IFN-NPA) After 30 Minutes of Incubation with the Different Media**

Release media	% IFN released
Effect of pH (Ionic strength=0.05 M)	
pH 5.0	35.7±0.9
pH 7.4	20.9±2.3
pH 8.5	8.81±0.2
Effect of ionic strength (pH=7.4)	
0.05 M	20.9±2.3
0.15 M	22.4±1.4
1.00 M	30.6±1.9

Data are expressed as the mean±s.d. (n=3).

TABLE 4 **NO Production by RAW Macrophages**

	NO production (μM)	
	Without LPS	With LPS
Basal	0.43±1.3	9.45±0.5
NP	0.71±1.3	12.8±0.7
IFN	25.1±0.95	33.5±1.8
IFN-NPA	30.7±2.1[a]	36.9±0.7
IFN-NPB	1.33±0.4	12.1±0.7

The cells were incubated in duplicate for 24 h with 20 ng/mL of IFN-γ free or loaded in nanoparticles, with or without LPS (100 ng/mL). Control NP represents the NO production by cells when incubated with an equivalent amount of albumin nanoparticles than used for IFN-γ nanoparticle formulations. Data are expressed as the mean±s.d. (n=3).

[a]$p<0.05$ vs. IFN-γ by analysis of one-way ANOVA with post-hoc Tukey's test applied for comparisons of several (≥ 3) group means.

DISCUSSION

Optimal delivery of pharmaceutical proteins must face up to a plethora of obstacles. Firstly, proteins and peptides exhibit poor physicochemical stability that compromise their integrity (Wang, 1999). To cap it, typical procedures for preparing nanoparticles as carriers for them implies manipulation conditions that are particularly aggressive (Cleland & Jones, 1996). Moreover, it is well known that upon surface interaction (mainly when it is mediated by hydrophobic forces), proteins undergo conformational changes that imply its denaturalization (Hlady & Buijs, 1996). In this context, it has been described that IFN-γ is indeed very sensitive to mechanical stress, like that caused by stirring of a protein solution, especially at concentrations lower than 1 μM (Zlateva et al., 1999). This manipulation reduces the Trp fluorescence by 75–80%, decreases significantly the α-helical content, and favors its aggregation. These effects seem to be mediated by surface-induced denaturalization (Boteva et al., 1996).

In spite of these previous reports about the high sensibility of IFN-γ to surface tension forces (Boteva et al., 1996), here we described conditions for preparing albumin nanoparticles, with exposure to the organic solvent ethanol, magnetic stirring for about

2 h, evaporation under reduced presure (once or twice depending on way for preparing IFN-loaded carriers) that had no effect on the fluorescence emission spectra and intensity of the cytokine (Fig. 1). It supports the claim that IFN-γ tertiary structure was not perturbed.

In the present work, as a way to load the IFN-γ to nanoparticles, we adsorbed the cytokine onto the surface of these carriers. As before mentioned, upon interaction with surfaces, the cytokine usually undergoes conformational changes, aggregation, and by this way inactivation. We could not directly observed the conformation of IFN-γ either once adsorbed onto the surface of nanoparticles or after desorption. However, the effects of pH and ionic strength indicated that the cytokine would be mainly adsorbed via electrostatic interactions (see Tables 1 and 3). In fact, the adsorption was maximal at pH ranged between *pI* albumin (about 5) and *pI* IFN (about 10), when the former has negative charge and the latter is positive (Table 1). Also, the desorption was higher when albumin molecules had no net charge (pH 5) (see Table 3). It has been described that ionic interactions have no significant effect on the stability of this cytokine (Hlady & Buijs, 1996). Moreover, the released IFN-γ fully retained its antiproliferative activity, as determined in Hela cells (Fig. 4). Similar behavior was observed when the cytokine was adsorbed onto multilamellar liposomes (van Slooten et al., 2001a). The inclusion of negative phospholipids in these formulations largely improved the association efficiency, dominated by electrostatic interactions (Ishihara et al., 1991; van Slooten et al., 2001a). Also, the interaction did not induce conformational changes and was released intact (van Slooten et al., 2000). As an advantage as compared with liposomes, albumin nanoparticles were able to load higher amounts of the cytokine, i.e., 1.23 μg/mg lipid in the best of cases (van Slooten et al., 2001a) vs. 2.5 μg/mg polymer (see Fig. 3); it has to be noticed it was the highest concentration tested for adsorption onto nanoparticles and we did not reach a plateau.

On the other side, when IFN-γ-loaded nanoparticles were prepared by a brief incubation between the IFN and albumin prior to the desolvation process and the cytokine was mainly entrapped in the matrix of the carriers, the system resulted inactive. In fact, IFN-γ was neither detected by ELISA nor bioassay (Fig. 4). Moreover, these carriers were also unable to stimulate macrophages for producing NO (Table 4). Differences between adsorption vs. encapsulation processes, in terms of the risk of cytokine degradation during the manufacturing process, are very subtle and thus, it is difficult to find an explanation for our observations. This procedure probably favors hydrophobic contacts (main forces linked to conformation perturbations and degradation for proteins) between albumin and the cytokine in compliance with more aggressive conditions. As a result, the cytokine seems to be released in an inactive conformation.

Once the risk of cytokine degradation during the process of formulation has gone beyond, IFN-γ meet other handicaps after their administration that limit its clinical use. To reach high IFN concentrations near macrophages, their main target cells, it is usually administered by a systemic route (the most frequent subcutaneous) in high concentrations. It yields significant side effects and toxicity, as fever, fatigue, nausea, vomiting, neurotoxicity, and leucopenia. With the association of the cytokine to nanoparticles, we can target IFN-γ in more concentration close to the macrophages. Thus, in accordance with its paracrine mechanism of action, IFN-γ associated must be more active than free. In fact, we found that IFN adsorbed to nanoparticles was slightly more potent for activating macrophages, measured as NO cleavage ($p<0.05$, Table 4). van Slooten et al. (2001a, 2001b) also noted that the adsorption of IFN-γ onto liposomes improved the ability of the cytokine to stimulate TNF and NO in mouse macrophage cultures. The authors related this superiority to a higher local density, which facilitates the interaction with its receptor (van Slooten et al., 2001a). So far, other authors indicated that IFN-γ must be leaked from the surface of liposomes to exert its activity (Eppstein et al., 1985; Fidler et al., 1985). In that sense, van Slooten et al. (2001a, 2001b) described a liposomal formulation with IFN-γ embedded into the bilayer of carriers that were less active than the free cytokine for in vitro activation of macrophages. Such disposition of the cytokine impaired its interaction with the receptor and subsequent activation signal.

In summary, the integrity of a therapeutic protein has to be safeguarded when formulated in delivery systems. In this study, we showed that the adsorption of the cytokine onto albumin nanoparticles could be a good strategy to both load IFN-γ and target the protein to macrophages. Under conditions that

preserved its conformation and activity, these carriers were able to load, mainly by electrostatic interactions, high amounts of the cytokine. So far, they improved the capacity of the free cytokine to trigger NO production in macrophages.

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