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Effect of levofloxacin and pefloxacin on humoral immune response elicited by bovine serum albumin docked in gelatin microparticles and nanoparticles

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The aim of the present investigation was to study the effect of levofloxacin and pefloxacin on the humoral immune response elicited by bovine serum albumin (BSA) encapsulated in gelatin particulate systems. FITC-BSA (Fluoresceine isothiocynate-bovine serum albumin) was entrapped in gelatin microparticles (GM) and gelatin nanoparticles (GN) prepared by emulsion polymerization and nanoemulsion methods, respectively. The prepared particulate carriers were evaluated for particle size, surface morphology, entrapment efficiency, zeta potential and *in vitro* antigen release. The optimized formulation of FITC-BSA loaded GM and GN were administered s.c. to albino rats and humoral immune response was measured in terms of systemic IgG antibody titre by ELISA method. The serum IgG response elicited was compared to that was obtained by s.c. administration of either free antigen or antigen emulsified (1:1) with Freund's in complete adjuvant (FIA). The vaccination of $2.41 \pm 1.56\,\mu$m sized GM elicited significantly ($P < 0.05$) higher serum IgG response than that obtained with administration of 107 ± 25 nm sized GN. Similarly, levofloxacin significantly ($P < 0.05$) decreased the antibody titre in rats immunized with BSA docked GM whereas pefloxacin did not reduce the antibody titre significantly. The study will help in programming a new drug management and in characterization of vaccine-drug interaction.

1. Introduction

Fluoroquinolones have been widely studied to establish the mechanistic of immunomodulation (Dalhoff 2005; Dalhoff and Shalit 2003; Madan et al. 2007) and proposed to interfere with cytokine production, which is an important signaling pathway in evoking of humoral and cellular immunity (Dalhoff 2005). The authors have investigated that ciprofloxacin interferes with the cellular components of the immune system and suppresses the humoral immunity in rats triggered by bovine serum albumin (BSA) encapsulated in niosomes and proposed that it could reduce the activity of IL-1β and IL-1α when the concentration of ciprofloxacin reaches 25 $\mu g.mL^{-1}$. Moreover, IL-1α present on mouse cell surface could play a major role in induction of humoral immunity. Similarly, chloramphenicol was also evaluated for its immunomodulation activity (Madan et al. 2007). Sequentially, the effect of other drugs like ampicillin and chloroquine was also investigated using BSA as model antigen (Madan et al. 2008). The immunomodulation activity of fluoroquinolones depends upon different parameters such as drug-plasma concentration, chemical structure and types of antigen. However, studies on immunomodulation by fluoroquinolones are still in their infancy and the methods applicable for its assessment are continually improving. For many of these agents, the underlying mechanisms of immunomodulation are not well defined and some events are strongly dependent on the method applied. On the other hand, particulate systems are better than traditional vaccine adjuvants. The adjuvants most frequently used for vaccines are mineral salts, aluminum compounds and water-in-oil emulsions such as Freund's complete and incomplete adjuvants. However, these immunoadjuvants have the disadvantage that they cannot be prepared in a physico-chemically reproducible manner (Jolles and Paraf 1973). Currently, aluminum hydroxide and aluminum phosphates are the only adjuvants that have been approved for use in humans but they cannot be lyophilized and are not effective for many vaccines (Bergstrand et al. 1983). However, it is well documented that controlled release of antigen is the only way of achieving a long-lasting instructive immune response. Hence, docking of antigen in a particulate system can release the antigen in a desirable and controlled fashion. Gelatin, a denatured collagen is capable of inducing long-lasting antigen release and also approved by FDA for human consumption (Nakaoka et al. 1995; Singh et al. 2002). The use of gelatin microparticles (GM) and gelatin nanoparticles (GN) as carrier of peptides, proteins and DNA vaccines require simple, easy to scale up technology, capable of high yield vaccine entrapment (Oya Alpar et al. 2005). Moreover, GM and GN can be engineered in a more reproducible manner than currently used vaccine adjuvants (Nakaoka et al. 1995; Won and Kim 2008). Not only the particle properties control the release of antigen but also size and surface properties of the particles have a great influence on adjuvant activity. Hence, controlling the size of gelatin particulate systems is an important parameter in induction of antibody titre. In

Table 1: Various process variables in preparation of FITC-BSA loaded gelatin microparticles

Sample (GM)	Concentration of gelatin (% w/v)	Amount of glutaraldehyde (25% v/v)	Encapsulation efficiency (%)	Mean diameter (μm)
GM1	20	0.4 mL	91.23 ± 2.63	15.38 ± 3.58
GM2	20	0.8 mL	88.72 ± 5.73	13.61 ± 4.17
GM3	15	0.4 mL	86.42 ± 4.18	9.48 ± 3.41
GM4	15	0.8 mL	82.62 ± 3.43	7.52 ± 2.42
GM5	10	0.4 mL	81.34 ± 6.33	5.20 ± 1.29
GM6	10	0.8 mL	78.66 ± 5.78	2.41 ± 1.56

(n = 3)

Table 2: Effect of glutaraldehyde concentration on particle size and entrapment efficiency of FITC-BSA loaded gelatin nanoparticles

Sample Code	Gelatin concentration (% w/v)	Glutaraldehyde concentration (25% v/v, μL)	Entrapment efficiency (%)	Size (nm)	Polydispersity index	Zeta potential (−mV)
GN1	0.025	15	55.47 ± 5.4	72 ± 15	0.095 ± 0.026	–
GN2	0.05	15	62.87 ± 6.7	80 ± 20	0.081 ± 0.009	–
GN3	0.025	10	69.49 ± 4.3	92 ± 17	0.085 ± 0.065	–
GN4	0.05	10	75.36 ± 5.6	105 ± 20	0.057 ± 0.010	–
GN5	0.025	5	74.24 ± 4.8	107 ± 16	0.098 ± 0.058	–
GN6	0.05	5	75.25 ± 3.2	107 ± 25	0.087 ± 0.049	−23 ± 0.4
GN7*	0.05	5	–	78 ± 10	0.089 ± 0.047	−15 ± 0.6

(n = 3)
* Blank gelatin nanoparticles

the present investigation, protein antigen, BSA was encapsulated in particulate systems (GM and GN) and the effect of fluoroquinolones such as levofloxacin and pefloxacin (selected as the model drugs) on the humoral immune response was evaluated. Therefore, an effort has been made to provide an inexpensive and sensitive method for the screening of fluoroquinolones for their immunomodulating activities.

2. Investigations, results and discussion

2.1. Preparation of FITC-BSA loaded gelatin particulate systems and its characterization

Gelatin has advantages compared to synthetic polymers in terms of natural origin, low immunogenicity, biocompatibility and biodegradability (Nakaoka et al. 1995). The FITC-BSA loaded GM was prepared by emulsion polymerization technique using glutaraldehyde as a cross-linker (Nakaoka et al. 1995). It was documented that GM of optimum size are capable of inducing systemic IgG immune response (Nakaoka et al. 1995). Hence, the primary objective of adopting emulsion polymerization method was to optimize the size of GM by varying the concentration of glutaraldehyde during the synthesis phase. Further, optical microscopy method was applied to measure the size of GM suspended in ether (to avoid the swelling of gelatin) revealed that every microsphere was spherical in shape, irrespective of the concentration of glutaraldehyde used for gelatin crosslinking. However, the size of the microparticles became smaller with the increasing concentration of crosslinking agent (Table 1), in good accordance with crosslinking theories (Karunakaran and Singh 1994). Subsequently, FITC-BSA loaded GN was engineered by nanoemulsion method using AOT-n-hexane-water system (Gupta et al. 2004). The particle size of GN was measured by a particle size analyzer (Malvern, UK) and indicated that narrow sized particles (72 ± 15 to 107 ± 25 nm) could be synthesized with low polydispersity index. However, the particle size of GN did not decrease ($P > 0.05$) as a function of glutaraldehyde concentration at low volume (Table 2). The surface morphology of GM and GN was observed by scanning electron microscopy (SEM), fluorescent microscopy (FM), transmission electron microscopy (TEM) and atomic force microscopy (AFM), respectively, which strongly imply that spherical and smooth shaped GM and GN have been engineered from gelatin (Fig. 1) using glutaraldehyde as a crosslinker and their size is consistent with the size, observed with optical microscopy (GM) and particle size analyzer (GN), respectively. The entrapment efficiency of GM and GN was determined in presence of collagenase enzyme (Table 1). The entrapment efficiency of GM decreased with increase in concentration of glutaraldehyde, which might be attributed to the fact that as the glutaraldehyde concentration increases the void volumes in microparticles decreased which further reduced the loading of FITC-BSA in GM (Katti and Krishnamurti 1999). However, varying the concentration of glutaraldehyde in preparation of GN could affect the entrapment efficiency of FITC-BSA only at higher concentration (Table 2). The zeta potential of FITC-BSA loaded GN (GN 6) was also reduced significantly ($P < 0.05$) in comparison of blank GN (GN7), which indicated the stability of FITC-BSA loaded GN in aqueous phase (Table 2). The *in vitro* release of FITC-BSA from GM (GM6) and GN (GN6) was carried out in presence of collagenase as shown in Fig. 2. The drug release profiles showed a biphasic modulation characterized by an initial relatively rapid release period, followed by a slower release period. However, the nanoparticles release the FITC-BSA at higher rate in comparison of microparticles. This might be attributed to the size of the GM (2.41 ± 1.76 μm) and GN (107 ± 25 nm) and as a result complete water penetration in GN (increase the surface area) supported by the faster enzymatic degradation and thus more free drug liberation (Mladenovska et al. 2002). Data of storage stability studies carried out for 3 months (Table 3) indicated that no significant ($P < 0.05$) change was observed, when optimized formulations (GM 6 and GN 6) were stored at 4 °C and 25 °C in comparison to 37 °C. Therefore, 4 °C and 25 °C are the optimum temperatures to store the FITC-BSA loaded GM and GN respectively.

2.2. Systemic IgG response

The effect of immunization of different formulations of BSA administered s.c. in rats on serum anti-BSA IgG response is

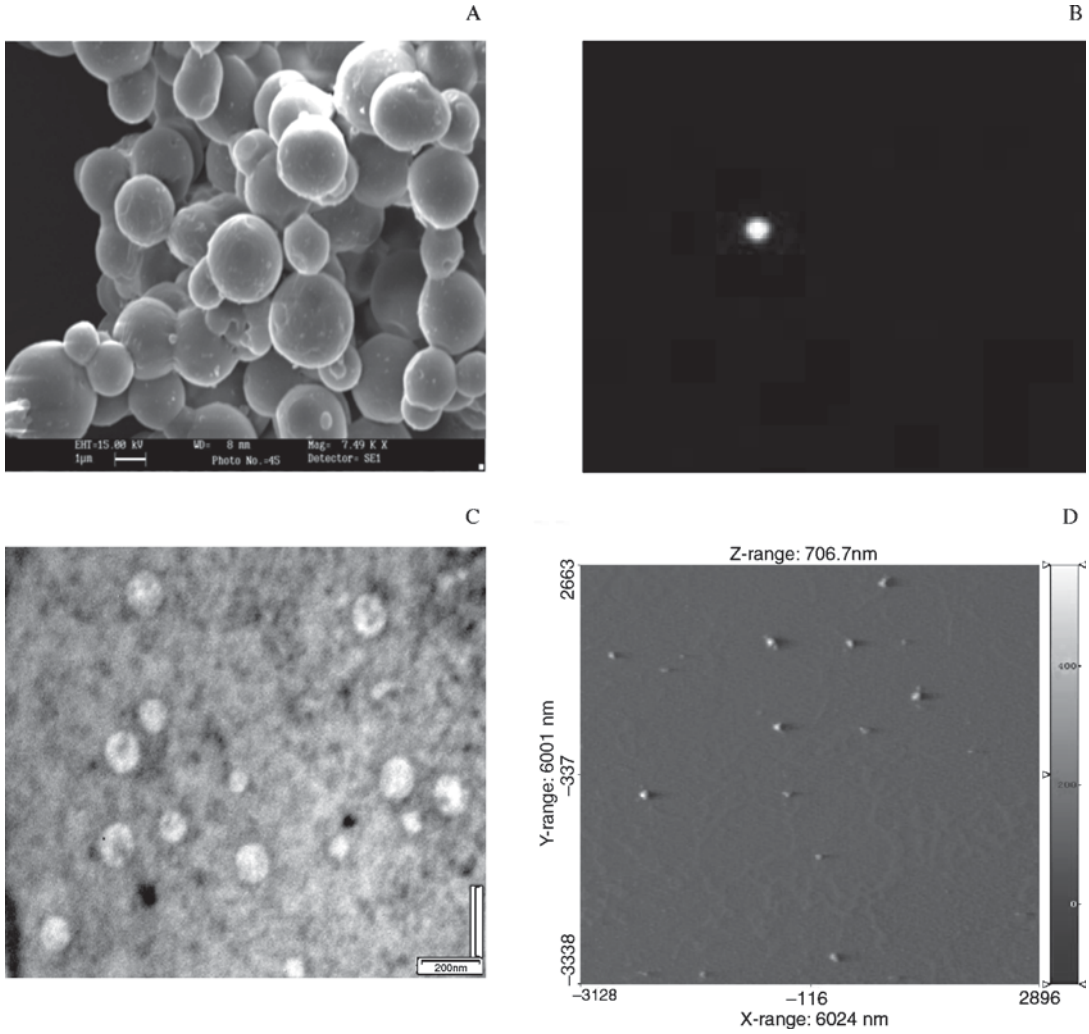

Fig. 1: (A) Scanning electron micrograph of BSA loaded gelatin microparticles (GM6), (B) Fluorescent photomicrograph of FITC-BSA loaded gelatin microparticles (GM6), (C) Transmission electron micrograph of BSA loaded gelatin nanoparticles (GN6) and (D) Atomic force microscopy of BSA loaded gelatin nanoparticles

shown in Fig. 3. It was observed that single s.c. dose of 100 µg equivalent of BSA entrapped in 2.41 ± 1.56 µm sized GM (GM 6) evoked an IgG response, which is insignificantly ($P > 0.05$) different from BSA + FIA formulation, used as positive control. However, the systemic IgG immunological response was significantly ($P < 0.05$) higher from BSA entrapped in GN (GN 6) of 107 ± 25 nm size. The 107 ± 25 nm sized GN also generated significantly ($P < 0.05$) higher antibody titre in comparison of BSA solution but lower compared to a single dose administration of BSA emulsified in FIA. Therefore, it has been proposed that microparticles (1–100 µm size) have a significant effect on immunological response. This might be attributed to the fact that differently sized antigen presenting polymeric decoys would have a quantitatively different distribution in the lymphoid tissue and there is an optimum size range for the phagocytosis of the particles by macrophages, which could be from 1 to 2.5 µm. These cells are well known for their ability to act as antigen presenting cells, so a better phagocytosis of 2.41 ± 1.56 µm sized GM could result in a better stimulation of the immunocomponents in order to evoke the systemic IgG response than that achieved with GN (Gutierro et al. 2002; Igattua et al. 1998; Nakaoka et al. 1995; O'Hagan et al. 1993; Tabata et al. 1991; Thomasin et al. 1996).

2.3. *Effect of levofloxacin on IgG antibody titre*

It has been investigated that levoflocxacin in therapeutic dose-dosage regimen (Table 4) significantly ($P < 0.05$) reduced the anti-BSA antibody titre in albino rats in two groups i.e. following

Table 3: Study of storage stability of the FITC-BSA loaded gelatin microparticles (GM6) and gelatin nanoparticles (GN6)

Parameters	Storage temperature (3 months)		
	4 ± 0.5 °C	25 ± 0.5 °C	37 ± 0.5 °C
Microparticle residual BSA (%)[a]	78.66 ± 5.78	77.28 ± 3.28	70.24 ± 4.29
Size	2.41 ± 1.56	2.27 ± 4.17	4.38 ± 2.98
Nanoparticle residual BSA (%)[a]	75.25 ± 3.2	75.12 ± 4.49	65.28 ± 1.17
Size	107 ± 25	106 ± 2.28	98.47 ± 3.36

(n = 3)
[a] Initially considered to be 100%

Table 4: Therapeutic dose-dosage regimen of levofloxacin and pefloxacin administered to rats

Drug	Dose	Duration of the course	Groups prepared for administration of the drug and BSA bearing gelatin microparticles
Levofloxacin	12.5 mg.kg^{-1} (OD, *i.m*)	5 days	Following medication (rats were medicated for 5 days and then immunized) During medication (rats were medicated for 2.5 days, then immunized and medicated again for the following 2.5 days) After 7 days of medication (rats were medi- cated for 5 days, then left to rest for 7 days and then immunized)
Pefloxacin	6.66 mg.kg^{-1} (BID, *i.m.*)	7 days	Following medication (rats were medicated for 7 days and then immunized) During medication (rats were medicated for 3.5 days, then immunized and medicated again for the following 3.5 adys) After 7 days of medication (rats were medi- cated for 7 days, then left to rest for 7 days and then immunized)
GM 6	100 μg	Single dose	Control group

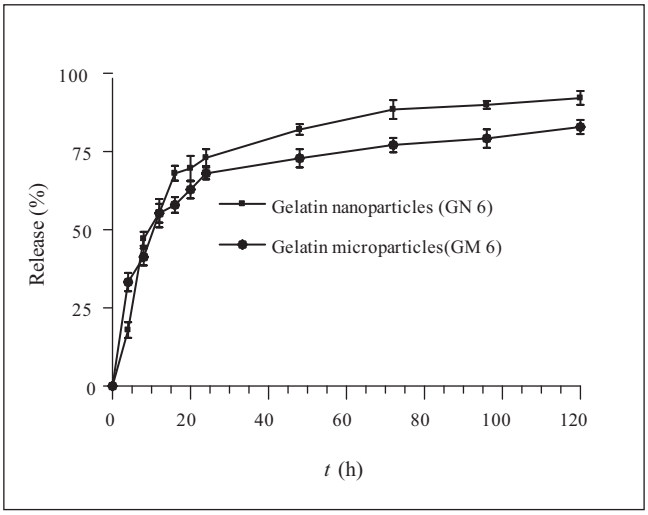

Fig. 2: *In vitro* release profile of FITC-BSA from different formulations of gelatin microparticles and nanoparticles in PBS (10 mM, pH 7.4) at 37 °C (n = 3)

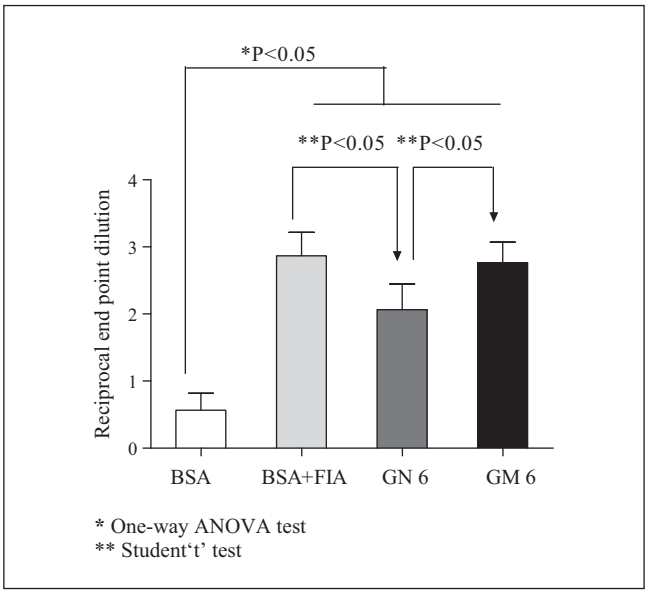

Fig. 3: The anti-BSA antibody titre produced in albino rats by inoculating BSA solution (BSA) or BSA + FIA or entrapped in gelatin nanoparticles (GN6) or gelatin microparticles (GM 6). Immunization was done through s.c. route. Plasma was sampled at 28 days of immunization at single dose (100 μg BSA). GM 6 produced significantly ($P < 0.05$) higher antibody titre in comparison of BSA or GN 6 (n = 6). ☐ BSA Solution; ▭ BSA+FIA (Positive control); ▬ GN6 (gelatin nanoparticles); ■ GM 6 (gelatin microparticles)

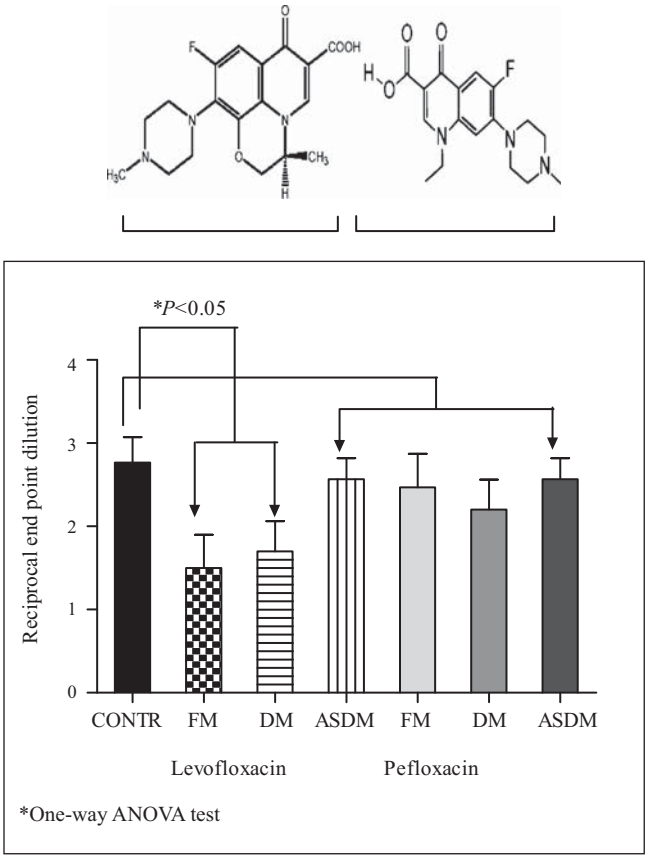

Fig. 4: Effect of levofloxacin and pefloxacin on humoral immune response in terms of anti-BSA antibody titre elicited by BSA loaded gelatin microparticles (GM6) Immunization was done through s.c. route. Plasma was sampled at 28 days of immunization at single dose (100 μg BSA). Levofloxacin significantly ($P < 0.05$) reduced the anti-BSA antibody titre in comparison of control group (without medication). However; pefloxacin did not reduce the antibody titre significantly ($P > 0.05$) (n = 6). ☐ Control (GM6); ▭ Following Medication; ▬ During Medication; ■ After Seven Days of Medication; ■ During Medication; ▩ After Seven Days of Medication

medication (FM) and during medication (DM) in comparison of control group (CONTR; GM6) as shown in Fig. 4. However, levofloxacin does not reduce ($P > 0.05$) the anti-BSA antibody titre in after seven days of medication (ASDM) group. Hence, the study indicated that levofloxacin suppresses the humoral immune response evoked by BSA-loaded GM (GM6). The immunosuppressive activity of levofloxacin might be attributed to the interaction of levofloxacin with immunocomponents such as T-cell population and cytokines, which are responsible for

the production of systemic IgG antibodies. It has been proposed that levofloxacin suppresses the IL-1β production, a key cytokine in T_H-cell activation (Yoshimura et al. 1996). Moreover, levofloxacin also suppresses the IL-2 level, which is normally produced by the body during an immune response. In contrast, levofloxacin does not have a cyclopropyl moiety (Fig. 4) at position N1 like ciprofloxacin, which is responsible for immunosuppressive activity. Therefore, levofloxacin suppresses the humoral immune response elicited by BSA bearing GM (Dalhoff 2005; Dalhoff and Shalit 2003; Madan et al. 2007).

2.4. Effect of pefloxacin on IgG antibody titre

It was observed that pefloxacin in therapeutic dose-dosage regimen (Table 4) did not reduce the anti-BSA antibody titre ($P > 0.05$) in rats in all three groups i.e. following medication (FM), during medication (DM) and after seven days of medication (ASDM) in comparison of control group (CONTR; GM 6) (Fig. 4). Hence, the data manifest that pefloxacin does not modulate the humoral immune response in rats elicited by BSA-loaded GM (GM6). This can be correlated with the previous investigations, which demonstrated that pefloxacin does not affect the humoral immune response triggered by EV vaccine fraction 1 antigen. Moreover, pefloxacin does not augment the IL-2 level. Therefore, pefloxacin does not interfere with the generation of instructive immune response (Smolkina 1995).
Hence, the present investigation may provide sufficient evidence that levofloxacin suppresses the humoral immune response when the rats were vaccinated with BSA loaded GM and peflocxacin had no effect on anti-BSA antibody titre in therapeutic doses. The study will help in programming a new drug management and in characterization of vaccine-drug interaction.

3. Experimental

3.1. Reagents and chemicals

Gelatin (Type B, bloom∼300, pH 6.3–9.2) and AOT surfactant were purchased from Sigma-Aldrich, USA. BSA and span 80 were purchased from Central Drug House, New Delhi, India. Glutaraldehyde (25% v/v solution) was obtained from S.D Fine chemicals, Mumbai, India. Levofloxacin (Glevo, i.v. Injection, 750 mg/150 ml) and pefloxacin (Pelox, i.v. Injection, 400 mg/100 ml 5% dextrose solution) were purchased from Glenmark (Majesta) Pharmaceuticals and Wockhardt Pharmaceuticals, India respectively. Fluoresceine isothiocyanate (FITC) was procured from Himedia, Mumbai, India. ELISA kit (Specific anti-rat IgG) and Freund's incomplete adjuvant were purchased from Banglore Genni, India. Collagenase was obtained as a gift sample from Central Leather Research Institute, Chennai, India. All other chemicals used were of analytical grade.

3.2. Conjugation of fluoresceine isothiocynate with bovine serum albumin

BSA (10 mg) was dissolved in 1 mL of distilled water, followed by adding 1% FITC (1:26 FITC to BSA). Then the conjugation reaction was carried out at 25 °C in an orbit shaker for 3 h. Different aliquots (5–800 $\mu g.mL^{-1}$) were prepared by diluting with distilled water. A calibration curve was prepared by measuring the fluorescence intensity using a UV spectrophotometer at 495 nm (Shimadzu 1601, Kyoto, Japan) (Li et al. 1999).
$Y = 0.0065 + 0.0149$, $R^2 = 0.9983$

3.3. Preparation of fluoresceine isothiocyanate-bovine serum albumin loaded gelatin microparticles

FITC-BSA loaded GM was prepared by emulsion polymerization technique using a chemical crosslinking agent (Nakaoka et al. 1995). Briefly, 0.2 mL of (10, 15 or 20%) gelatin solution (aqueous phase) containing 10 mg. mL^{-1} of FITC-BSA was added to 5 mL of a chloroform/toluene (1:1 v/v) mixture (oil phase) containing 2% w/w span 80 as a surfactant. The mixture was sonicated for 10 min (Soniweld, India) to prepare a water-in-oil emulsion. Subsequently, glutaraldehyde (25% v/v solution) (0.4 mL or 0.8 mL) was added to the emulsion as a crosslinking agent. The crosslinking reaction was allowed to proceed in ice-cold conditions for 6 h. The resulting GM were filtered, washed with isopropyl alcohol and phosphate buffer saline (PBS, 10 mM, and pH 7.4) and finally suspended in PBS.

3.4. Preparation of fluoresceine isothiocyanate-bovine serum albumin loaded gelatin nanoparticles

FITC-BSA loaded GN was prepared by nanoemuslion method (Gupta et al. 2004). To 50 mL of 0.03 M AOT in n-hexane solution, 800 μL of 0.025 or 0.05% w/v of gelatin aqueous solution was added. Simultaneously, 5–15 μL (25% v/v solution) of glutaraldehyde was added to this solution to synthesize the nanoparticles. 20 μL of FITC-BSA solution (35 mg. mL^{-1} in distilled water) was added to entrap the antigen in GN. The GN were then recovered from nanoemulsion by evaporating the n-hexane in a rotary flash evaporator (Buchi, Switzerland) and nanoparticles from remaining dry mass were recovered by precipitation in an excess of acetone-methanol mixture (9:1) followed by filtration. The precipitate was washed three times with an acetone-methanol mixture and resuspended in PBS (10 mM; pH 7.4) followed by dialysis using 12 kD cut-off dialysis membrane (Sigma, USA) against double distilled water. The aqueous suspension of nanoparticles was then lyophilized to obtain dry powder.

3.5. Particle size and shape characterization of microparticles and nanoparticles

Particle size of the GM was analyzed by optical microscopy (Karunakaran and Singh 1994) using a compound microscope. An ethereal suspension of GM was allowed to dry on a clean slide to form a thin film of particles and the size was measured for each batch using a calibrated ocular micrometer. The arithmetic mean diameter (AMD) was calculated according to the formula:

$$AMD = (n_1 d_1 + n_2 d_2 + \_ \_ \_ + n_m d_m)/(n_1 + n_2 + \_ \_ \_ + n_m) \quad (1)$$

where n_1, n_2, . . ., n_m are the number of particles and d_1, d_2,. . .,d_m are the diameter of the particles. For GN, 100 μL of GN suspension was diluted to 4 mL with PBS (10 mM; pH 7.4) and size was measured in a particle size analyzer (Malvern Instruments, UK). The shape and surface morphology of the GM was investigated using scanning electron microscopy (SEM; Jeol, JSM-6100). The GM were fixed on supports with carbon glue and coated with gold using a gold sputter module (JFC-1100) in a high vacuum evaporator. Samples were observed by SEM at 15 kV. The fluorescent image of FITC-BSA loaded GM was observed on a fluorescent microscope (Nikon Eclipse E 600). On the other hand, size and shape of the GN were examined using a Philips Morgagni 268 transmission electron microscope at a voltage of 80 kV. The aqueous dispersion of the GN was drop cast onto a carbon coated copper grid and grid was air dried at room temperature before loading in to the microscope. The shape and surface morphology of GN was also investigated using atomic force microscopy (Molecular Imaging, USA). Imaging was done in air using 0.7 μm AFM head. The sample was placed on xyz-piezo-translator and scanned by using a sharp diamond tip mounted on a gold coated 200 μm triangular Si_3N_4 microfabricated cantilever (force constant = 0.6 N/m). The force between the tip and sample usually ranges from 10^{-7} to 10^{-9} N.

3.6. Determination of zeta potential

The surface charge of GN was determined by laser Doppler anemometry using a zetasizer (Malvern Instruments, UK). For the measurement, 100 μL of GN suspension was diluted to 4 mL with PBS (10 mM; pH 7.4) solution. An electric field of 150 mV was applied to observe the electrophoretic velocity of the GN. All the measurements were made at 25 °C in triplicate.

3.7. Determination of entrapment efficiency

FITC-BSA loaded GM and GN (10 mg) were dispersed and digested in 10 mL of PBS (10 mM; pH 7.4) with collagenase (20 U/mL) and incubated in an orbit shaker at 200 rpm for 24 h at 37 °C. The amount of FITC-BSA released from the particulate systems was diluted appropriately with PBS (10 mM; pH 7.4) and quantified using UV spectrophotometer (Shimadzu 1601, Kyoto, Japan) at 495 nm.

3.8. In vitro antigen release

FITC-BSA loaded GM and GN (10 mg) were placed in 50 mL of conical flasks containing 30 mL of PBS (10 mM; pH 7.4) with 0.8 μg. mL^{-1} collagenase. The conical flasks then fitted to an orbit shaker maintained at 50 rpm and 37 °C. The samples were withdrawn at predetermined time intervals (2, 4, 6, 8, 10, 12, 24, 48, 72, 96 and 120 h) and replaced with PBS (10 mM; pH 7.4) to maintain infinite sink conditions. The process was repeated thrice and the samples were analyzed for FITC-BSA at 495 nm (Gutierro et al. 2002).

3.9. Stability study on storage

The ability of GM and GN to retain the antigen was assessed by keeping the GM and GN in sealed vials (10 mL capacity) after flushing with nitrogen at 4 ± 0.5, 25 ± 0.5 and 37 ± 0.5 °C for three months under controlled conditions (dark) and then evaluated for their size and leaked percent of encapsulated FITC-BSA at 495 nm. The initial antigen content was considered as 100%.

3.10. Medication and immunization

In vivo studies were carried out as per the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Culture, Government of India. The Institutional Animal Ethics Committee approved the study. Male albino rats (Wistar strain, 80 ± 20 g) were maintained at standard laboratory chow and water *ad libitum* in a temperature and light controlled environment at Hindu College of Pharmacy, Sonepat, Haryana, India. Rats were divided into 10 groups of six animals each. Firstly, four groups received 100 μg equivalents of BSA s.c. in form of BSA solution (BSA in 10 mM PBS, pH 7.4), BSA emulsified with FIA (BSA + FIA), BSA encapsulated in GN (GN6) and BSA in GM (GM6). Secondly, six groups received levofloxacin and pefloxacin injected i.m. as per the therapeutic dose-dosage regimen based on the body mass of rats (Table 4). Simultaneously, levofloxacin and pefloxacin-treated rats were immunized s.c. with a single dose of 100 μg equivalent of BSA encapsulated GM (GM6), as the 100 μg of BSA encapsulated in colloidal carriers induced significantly higher antibody titre (Brewer and Alexander 1992). Immunization was done after pre-medication of albino rats with levofloxacin and pefloxacin applying three variables-following medication (FM), after seven days of medication (ASDM) and during medication (DM). The immune response of BSA-docked GM (without medication) was designated as the control (CONTR) group.

3.11. Determination of IgG titre by ELISA

Specific anti-BSA antibody level in the serum was determined by ELISA (Brewer and Alexander 1992). Each well of the flat bottomed polystyrene plate was coated with 100 μL of BSA (1 mg L^{-1} in 0.02 mol L^{-1} Tris/HCl, pH 9.0) and incubated overnight at 4 °C. Thereafter, the wells were washed ten times with PBS-Tween buffer (0.02 mol L^{-1} Tris, 0.05% v/v Tween 20, pH 7.4) and to each well, 100 μL of the diluted serum sample was added and incubated for 2 h at room temperature. The plates were washed three times with PBS-Tween buffer and 100 μL of diluted horseradish-peroxidase-conjugated anti-globulin specific anti-rat IgG was added to each well and incubated for 2 h. The plates were again washed three times with PBS-Tween buffer and 100 μL of substrate solution 3,3',5,5' tetramethyl benzidine containing hydrogen peroxide was added to each well. The plates were incubated in the dark at room temperature for 15 min. The reaction was stopped by adding 50 μL of 2 mol L^{-1} H_2SO_4 to each well and the absorbance was measured at 450 nm using a microplate ELISA reader (Lab system Multiscan, Finland). The immune response was shown in terms of reciprocal end point dilution.

3.12. Statistical analysis

The results were expressed as mean ± standard deviation. Statistical analysis was carried out using one–way ANOVA and *t* tests. All statistical calculations were performed with GRAPH PAD Prism 4.01 software.

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