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Immune Response to Nasal Delivery of Antigenically Intact Tetanus Toxoid Associated with Poly(L-lactic acid) Microspheres in Rats, Rabbits and Guinea-pigs

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Abstract—Tetanus toxoid was adsorbed onto poly(L-lactic acid) microspheres. Analyses by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blotting suggest that the formulation procedure does not affect the stability or the antigenic properties of the protein. After nasal administration to guinea-pigs, the resulting preparation enhanced the immune response to the tetanus toxoid when compared with the free antigen. The increase in systemic immunoglobulin G titre was almost immediate in the group treated with the adsorbed tetanus toxoid (time zero, 140; week 2, 1550; week 4, 2760), reaching 36 000 two weeks after the booster (week 7), whereas the free antigen produced an immune response similar to that found in non-treated animals. In a parallel experiment, latex particles of a similar size to poly(L-lactic acid) microspheres, administered to both rats and rabbits, were detected in the blood stream. These findings with tetanus toxoid demonstrate the use of microsphere delivery systems for nasal application of antigens. These had a profound effect on the immune response, and indicate possibilities for overcoming some of the barriers to drug absorption in general.

The exploitation of new generation vaccines or delivery of peptides and proteins in general has been impeded by a lack of appropriate delivery systems. Most rDNA, peptide and protein molecules show poor transport characteristics across the epithelial barrier and are most commonly administered parenterally, but an alternative route would be preferable. Due to its easier accessibility, the nasal cavity is a potential alternative to the parenteral route for peptide drugs. On the other hand, although nasal and pulmonary absorption routes are potentially important in the administration of immunogenic substances, few reports have indicated their full potential (O'Hagan & Illum 1990).

Many investigators have confirmed that for certain infectious diseases the mucosal route is the most appropriate way of immunization (Bienenstock 1988; Eldridge et al 1990, 1991). The antigens may be administered locally to the highly concentrated immune active tissues known as the bronchus-associated lymphoid tissue (BALT) or to the nasal-associated lymphoid tissue (NALT) (Spit et al 1989) at certain areas of the respiratory tract for the induction of an immune response. Nasal administration of antigens could produce effective vaccines, both for protection of the upper respiratory tract and for potentiation of systemic immunity. It is already well documented that the main function of BALT, like GALT (gut-associated lymphoid tissue), is the selective absorption and uptake of antigens, and the induction of local immune response (Wolf & Bye 1984; McCluggage et al 1986; Eldridge et al 1989).

Antigens that are responsible for the induction of protective immunity against various pathogens may be attached to a suitable carrier with adjuvant (or sustained release) properties to induce effective and long lasting local immunity by interacting with mucosal-associated lymphoid tissue, or for inducing protective systemic immunity upon reaching immune competent organs, such as spleen, against systemic infections (Gregoriadis 1990). The paucity of information in the area of nasally administered, carrier-associated antigens, stimulated our interests to investigate further the advantages of using this route.

In the present work, we have immunized guinea-pigs by nasally administering tetanus toxoid (TT). The experiments were designed to study the nasal route for delivering antigens associated to particulate carriers for inducing immune reactions. In recent years much has been learnt about microspheres and nanospheres as drug carriers. Microspheres prepared with poly(lactic acid) (PLA) and poly(lactide-co-glycolide) have been used to deliver antigens (O'Hagan et al 1989a; Eldridge et al 1990, 1991). In this study, in contrast to the usual encapsulation system, we have used an adsorption procedure to bind the antigen to the PLA microspheres.

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Materials and Methods

Materials

Poly(L-lactic acid) (mol. wt 2000 Da) was obtained from Polysciences, Inc., Northampton, UK. Tetanus toxoid was obtained by dialysis and freeze-drying of a preparation of Tetanus Vaccine BP (in simple solution) purchased from The Wellcome Foundation (Beckenham, UK). Polyvinyl alcohol (PVA; mol. wt 10 000 Da, 88% hydrolysed; Aldrich Chemical Company, UK) and methylene chloride (Fisons, Loughborough, UK) were also used. Latex particles (fluoresbrite carboxylated microspheres, 0·83 μm $\pm$ 0·03 and 0·51 μm $\pm$ 0·008, mean size $\pm$ s.d.) were obtained from Polysciences, Inc., Northampton, UK.

Animals

Female Dunkin-Hartley guinea-pigs, $\approx$ 300 g, were used in the immunization experiments. Male Wistar rats, $\approx$ 400 g,

and New Zealand White rabbits, ≈3·5 kg, were used for the determination of nasal uptake of microspheres. All animals were allowed free access to food and water during these experiments.

Microsphere preparation and characterization

The PLA microspheres were prepared by using a modification of a solvent evaporation method (Benita et al 1984). A solution of 5% PLA in methylene chloride was mixed with an aqueous phase consisting of 1·5% polyvinyl alcohol in water, under vigorous stirring (Silverson Mixer, Silverson Machines Ltd, Chesham, UK), both phases being previously cooled in an ice bath. The mixture was stirred until the organic solvent completely evaporated. The particles were obtained after washing with distilled water, centrifuged and freeze-dried. Particle examination was performed by scanning electron microscopy (SEM; Cambridge Instruments Stereoscan 90). Particles were sized in micrographs using the micron bar as a reference. The data were plotted as histograms and the particle size distribution analysed.

Protein adsorption

Microspheres were incubated with a solution of protein in saline, for 24 h. The degree of adsorption was calculated indirectly by determining the amount of protein remaining in the supernatant after centrifugation. The supernatants were assayed by the bicinchoninic acid protein assay (Smith et al 1985). The adsorption procedure was also monitored by carrying out SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) of starting solutions, supernatants and washes. The amount of protein adsorbed onto the particles was also determined by digesting the microspheres with 0·1 M NaOH containing 5% SDS (Hora et al 1990). The resultant solution was adjusted to pH 7 with 0·1 M HCl, and assayed for protein.

Release characteristics

These were monitored over time by assaying 1 mL portions of dissolution medium (at 37°C), which was 20 mM phosphate buffer, pH 7·5, by the protein assay and also checked by SDS-PAGE.

Assessment of protein integrity

The assessment of TT integrity was investigated using SDS-PAGE and immunoblotting techniques. Protein samples were analysed before adsorption and after release from the microspheres, using a 12% gel and the Mini-Protean system (Bio-Rad, Watford, Herts) according to the method described by Lugtenberg et al (1975). The immunogenic properties of the antigen molecule after adsorption and release were measured in-vitro by immunoblotting (Western blotting) (Towbin et al 1979). The blots were visualized using 4-chloro-1-napthol (Sigma Chemical Co., Poole, UK).

Immunization protocol

Three groups of guinea-pigs (n=3) were used in these experiments. One unique dose of 60 μg of TT was used, group 1 being treated with microsphere-associated TT, group 2 with free TT dissolved in isotonic phosphate-buffered saline, pH 7·4 (PBS) and group 3 (control) was given only buffer solution. Vaccine was obtained by suspending the microsphere preparation in a certain volume of vehicle in order to obtain the required concentration of TT. This preparation was carried out just before administration. Simultaneously, a solution of free TT in the same vehicle and of the same concentration was prepared. Animals were given the vaccine by introducing 100 μL of the appropriate preparation into each nostril by means of a micropipette.

Measurements of the immune response

Antibody response to the TT in immunized animals was monitored by the microplate ELISA technique of Davis & Gregoriadis (1989). TT (5 μg mL^{-1}) in 0·05 M sodium carbonate-bicarbonate buffer (pH 9·6) was added to Dynatech microelisa plates and incubated overnight at 4°C. The wells were washed three times with 9·5 mM sodium phosphate buffer containing 0·8% sodium chloride and 0·05% Tween 20 (PBST), pH 7·4. Dilutions of serum from 1:50 in PBST + 0·1% bovine serum albumin (Sigma, UK) were incubated in plates for 2 h at room temperature (21°C). The total amount of bound immunoglobulin G (IgG) was estimated by the addition of a specific anti-IgG-horseradish peroxidase conjugate. Anti-tetanus immunoglobulin (Humotet, The Wellcome Foundation Ltd, UK) was used as a standard. Protein A peroxidase was used to estimate the total amount of IgG bound to the TT. After 1 h incubation at room temperature the enzyme substrate 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma, UK) was added. The reaction was stopped by the addition of 2 M sulphuric acid and absorbance at 450 nm measured (Anthos Reader 2001). The endpoint of each titration was defined as the dilution at which the absorbance was 0·2 (instrument zeroed on the reagent blank). This was expressed as a titre correction by reference to a standard on every plate.

Nasal administration of non-biodegradable latex particles

Particles with a mean diameter of 0·51 and 0·83 μm suspended in saline were administered to rats and rabbits respectively, intranasally, using a Gilson pipette. The volume administered was 0·1 mL for rats (0·05 mL per nostril) and 0·2 mL for rabbits (0·1 mL per nostril).

Blood samples

Blood samples for particle and erythrocyte counts were taken from the tail vein of rats and from the ear vein of rabbits. Counts were made using a haemocytometer with a microscope under both UV and tungsten light.

Results

Transfer of particles from the nasal cavity to the circulation

Male Wistar rats (n=4) were dosed intranasally with $1{\cdot}38 \times 10^{10}$ particles, mean diameter 0·51 μm ± 0·008 suspended in saline. The mean values for particles counted per 100 erythrocytes in blood samples were calculated and expressed as percentage of total administered particles (Fig. 1). An example of the calculations is as follows: erythrocyte counts on the rats per mL gave a mean value of $7{\cdot}0 \times 10^9$ cells. Since the blood volume of a rat is 5 mL per 100 g of body weight (Green 1982) a value of 1 particle per 1250 erythrocytes would represent about 0·96% of the administered particles in the circulation, 10 min after dosing.

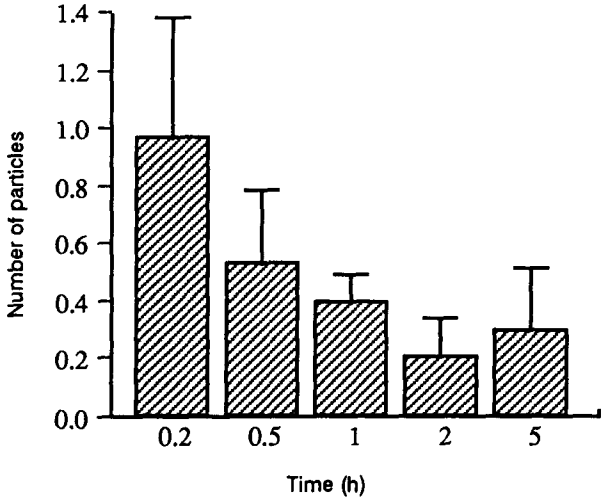

FIG. 1. Levels of circulating fluorescent latex particles (mean size 0·51 μm $\pm$ 0·008) after nasal administration to rats (n = 4). Particle numbers were calculated as described in the text. Error bars represent s.d.

Four rats were individually dosed with latex particles of size 0·51 μm $\pm$ 0·008. Ten minutes after dosing, the numbers of particles in tail vein samples were found to be 0·96% ($\pm$0·42, s.d.) of the number administered; at 30 min, 0·53% ($\pm$0·25); at 1 h 0·39% ($\pm$0·10); at 2 h 0·20% ($\pm$0·14); at 5 h 0·29% ($\pm$0·22); and at 24 h < 0·14% (Fig. 1). This experiment demonstrated a fast transfer of particles to the circulation, but a cut-off point may exist for larger particles.

Circulating levels of particles were expressed from free circulating particles. In some of the preparations, particles accumulated in circulating macrophages were also detected.

The experiment was repeated with rabbits (n = 2), which received particles with a diameter of 0·83 μm $\pm$ 0·03, suspended in saline. At 10 min the blood samples contained 1·9% of particles (range: 2·9–1·0); at 60 min 3·4% (range: 5·2–1·5) and at 2 h 1·7% (range: 2·7–0·7) of administered particles. At 24 h, particles were present (0·5%) in the circulation. Rabbits were dosed to check that nasal absorption of particles occurred in species other than rat. Rats were chosen for the convenience of blood sample collections.

Vaccine characterization

The method of preparation of PLA microspheres produces spherical microspheres with an apparently smooth surface (Fig. 2). Particle size is distributed around a mean size of 0·8 μm with a range of 0·1–1·6 μm (Fig. 3).

Adsorption of TT on to PLA microspheres was determined indirectly by assaying the supernatant following centrifugation of the microsphere suspension and directly after extraction of the protein by the digestion method (Hora et al 1990). The results were concordant and the value for the amount of protein adsorbed was 39·3% (w/w) of the initial amount of TT, which is equivalent to 1% (w/w) loading in the final preparation. The SDS-PAGE method was also used for the control of the adsorption procedure and confirms these results (Fig. 4). Our data closely fits the Langmuir type of isotherm (correlation 0·993).

The release of adsorbed protein from microspheres was also the subject of study. Fig. 5 shows that approximately

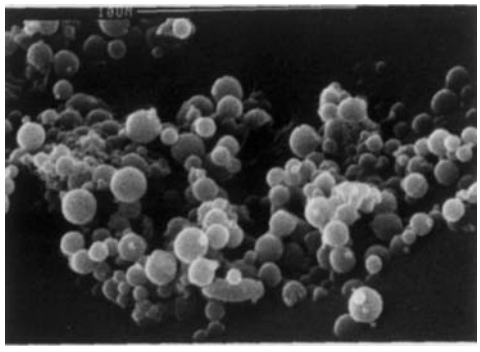

FIG. 2. SEM of PLA microspheres prepared by the modification of the solvent evaporation method.

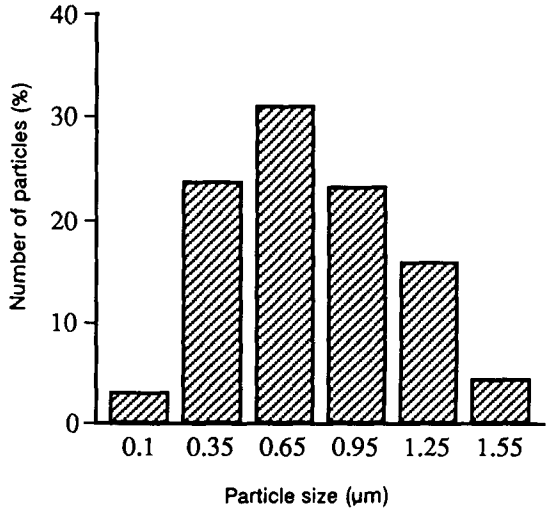

FIG. 3. Particle size distribution of PLA microspheres as measured from slides obtained by scanning electron microscopy (mean size = 0·8 μm; n = 231 particles).

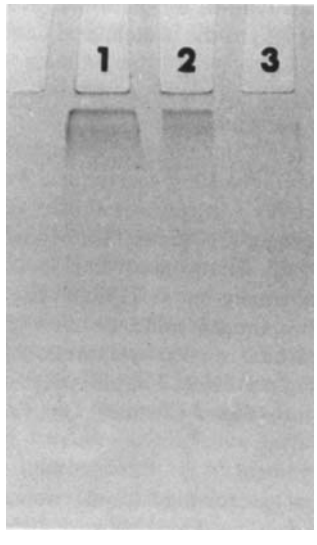

FIG. 4. SDS-PAGE (12% gel, stained with Coomassie blue) of TT before and after adsorption on to PLA microspheres. Lanes: (1) Tetanus Vaccine BP in Simple Solution; (2) TT solution 250 μg mL^{-1} before adsorption; (3) TT solution 250 μg mL^{-1} after adsorption (supernatant).

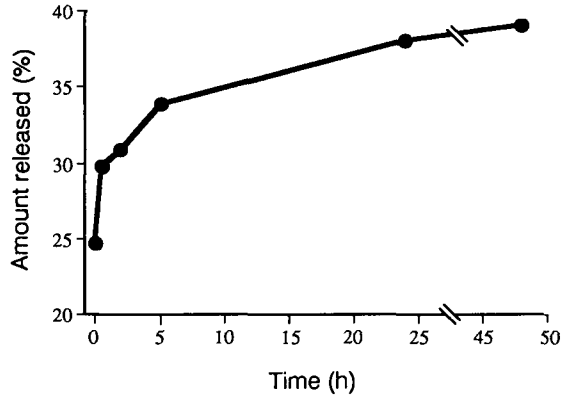

FIG. 5. Release profile of tetanus toxoid adsorbed on to PLA microspheres in 20 mM phosphate buffer, pH 7·5.

40% of the adsorbate is released within 48 h. These results compare favourably with those quoted in the literature, since proteins are large molecules with a large number of binding sites on to the microsphere surface that are not likely to desorb simultaneously (Norde 1984) resulting in a slow release profile.

Stability of the antigen

After release of TT from microspheres, the stability of the antigen was investigated by SDS-PAGE (Fig 4) and showed that the structure of the molecule was maintained. The sorption procedure, although described as affecting some properties of the peptidic molecules (Norde 1984) does not affect the whole structure of TT. We have also found by immunoblotting that the antigenicity of this protein is not affected (Fig. 6).

Immune response

Animals were immunized with TT (free or PLA microsphere associated) and circulating anti-tetanus IgG was assayed by

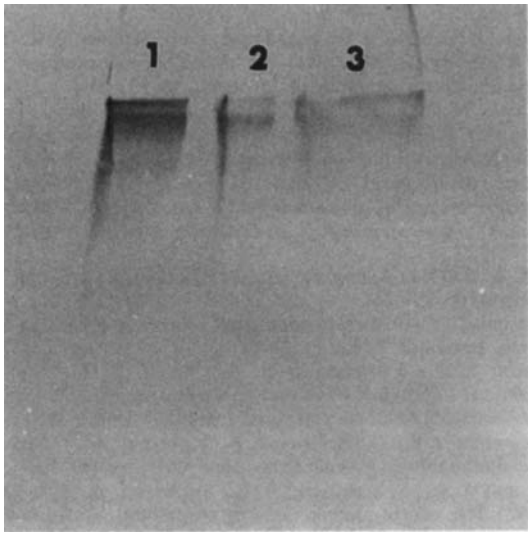

FIG. 6. Western blotting of TT before and after adsorption on to PLA microspheres. Lanes: (1) Tetanus Vaccine BP in simple solution; (2) TT solution before adsorption; (3) TT after release from PLA microspheres under the conditions described in text.

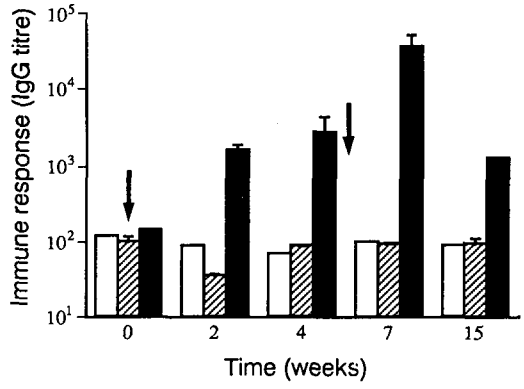

FIG. 7. Serum antibody response to nasally administered TT (mean ± s.e.; n = 3). Animals were given the vaccine by nasal administration of 60 μg of free or microsphere-adsorbed toxoid. Priming consisted of three doses on days 1, 2 and 3, respectively, and booster consisted of one dose one month after the third dose of priming (week 5).

the ELISA method described above, in blood samples collected at weeks 2, 4, 7 and 15. The increase in IgG titre was almost immediate in the group treated with the adsorbed TT (time zero, 140; week 2, 1550; week 4, 2760), reaching 36 000 two weeks after the booster (week 7), whereas the free antigen produced an immune response similar to those found in non-treated animals (control, given PBS only). On the other hand, at week 15 (10 weeks after the booster) the titre produced by the microsphere preparation had decreased to 1200, which still is 8·5 times higher than the pre-bleed value of 140 (Fig. 7).

Discussion

The results indicate that association of TT with PLA microspheres through our stable adsorption procedure has increased systemic IgG responses to nasally delivered antigen over free antigen delivered in solution. It is now accepted that the form of an antigen determines the immune response. Thus, it is important to evaluate suitable adjuvant/antigen combinations which are administered mucosally but still increase the serum antibody (or mucosal antibody) levels according to the type of infection or type of protection required. Increased saliva and serum antibody levels have been observed following oral administration of various soluble antigens with or without co-adjuvant (muramyl dipeptide) delivered as colloidal systems when compared with free antigen delivery (Wachsman et al 1985; O'Hagan et al 1989a, b; Eldridge et al 1990). There have been few previous studies using the nasal route (El Guink et al 1989) for the administration of particulate vaccines. In this study we have shown that the nasal administration of PLA-associated TT gave higher antibody response than that from the administration of the same quantitiy of free antigen.

Maximal IgG titres occurred in serum 40 days after the priming. The primary response lasted about 20 days, but even after 30 days the titres did not reach the pre-immune levels, i.e. it was not a short-lived response, considered to be the main disadvantage of mucosal immunization. The secondary response was rapid (occurring only 10 days after a single delivery) suggesting that our mode of immunization was stimulating serum IgG memory.

The observed serum IgG antibody production stimulated by particulate TT may indicate an interaction of TT with non-NALT (Spit et al 1989) lymphatic tissues, such as spleen, after being drained from the lymphatic vessels in the nasal cavity. This would imply that uptake and transfer of particles through the nasal mucosa had occurred: indeed, we have shown that latex particles have been taken up following nasal administration. Also, the particulate nature of antigens may give rise to enhanced immune responses after interaction with immunocompetent cells. The data agree with the findings of Perkins et al (1969) who stimulated both secretory IgA and serum antibody production by the intranasal administration of killed rhinovirus.

Macromolecular uptake is well documented (Warshaw et al 1971; Richardson et al 1976), as is that concerning the non-carrier nasal administration of antigens (Fontages et al 1980; Wood et al 1983). However, fewer citations are available on methods which rely on a carrier such as microspheres.

Our results show that particles (average size of 0·51 μm and 0·83 μm) when administered nasally can penetrate into the blood circulation and therefore possibly into the spleen. No attempt was made to enhance the absorption or to prolong particle longevity by precoating with agents (Davis & Illum 1986) to produce a hydrophilic surface. Since the counts are carried out only on the circulating particles, a surface coating process would maintain higher particle concentrations for longer periods of time so producing a more sustained immune response by the immunocompetent cells possibly due to a more gradual removal of particles by the reticuloendothelial system. These results agree with those in which we delivered latex particles orally (Alpar et al 1989), when there was rapid absorption from the gastrointestinal tract and transport into the circulation. The mode of entry of orally administered particles into the circulation is unknown and few investigators have published (Jani et al 1990) on putative mechanisms. Thus, the induction of the immune response also remains obscure although in the gut, antigens, pathogens and particles are taken up by M-cells in the GALT (Wolf & Bye 1984; McCluggage et al 1986). In the respiratory area, where the more complex mechanisms are involved, it has been shown that lymphoepithelial cells of BALT are also involved in antigen sampling and uptake (Van der Brugge-Gamelkoorn et al 1985; Gregson et al 1982). Many similarities between the lymphoid follicles of BALT and GALT suggest similar fundamental mechanisms may be involved in the uptake process.

Scavenging of particles and pathogens by macrophages into immunocompetent cells has also been suggested as a route of absorption (Tenner-Rácz et al 1979; Corry et al 1984; Harmsen et al 1985). Tonsils, being situated near the junction of the respiratory and upper alimentary tract are exposed both to air and to swallowed particles and may represent an integration site between peripheral and mucosal immune systems (McDermott et al 1982).

Irrespective of the mode of entry, our findings concerning the uptake of particulate carriers by the nasal route suggest that the nasal mucosa is a potential administration route for delivery of particulate vaccines.

Microspheres apparently possess adjuvant properties when introduced by the subcutaneous intramuscular, and oral routes (Eldridge et al 1990, 1991; O'Hagan et al 1989a, b). The present study also suggests that nasally administered particles also possess adjuvant properties possibly due to their ability to act as depot systems.

On the basis of our results, the use of microspheres as novel nasal immunization agents appears to be promising.

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