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Immunological consequences of nasal drug delivery in dextran microspheres and ethyl(hydroxyethyl)cellulose in rats

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Abstract

These experiments were performed to evaluate the immunological implications of intranasal drug delivery in dextran microspheres and a thermogelling ethyl(hydroxyethyl)cellulose (EHEC) system. The amount of IgA and IgG in plasma and nasal washings from rats administered with equine myoglobin in the two nasal delivery systems were not significantly different from the controls throughout the experiment. The intranasal administration generated neither specific IgA nor IgG antibodies in plasma or in nasal washings. When the animals were boosted intramuscularly with Freund's complete adjuvant on day 26 they produced a plasma IgG response similar to the response in animals that received one intramuscular injection of equine myoglobin in Freund's complete adjuvant on day 1. The conclusion is therefore that dextran microspheres and EHEC do not act as adjuvants, potentiating the immunogenicity of heterogeneous proteins. This is an important property for a nasal drug delivery system to fulfil.

Keywords: Nasal administration; Dextran microspheres; Thermogelling polymer; Immunology

1. Introduction

One of the major functions of the nasal airway is to protect the body from inhaled hazardous materials. The nasal mucosa are the first site of contact with inhaled antigens and such exposure can provoke both local and systemic immune response (Kovatchev et al., 1990). To induce an immunological response the antigen has to be presented to lymphoid tissue containing antibody-producing cells. In the context of mucosal vaccination an adjuvant for mucosal application is usually needed to accomplish mucosal immunity as well as systemic response. It has been shown that cholera toxin when administered intranasally concurrently with influenza HA vaccine stimulated the antiviral IgA response in nasal secretions (Tamura et al., 1988). Intranasally delivered liposomes can stimulate mucosal immune response against the incorporated protein BSA (Aramaki et al., 1994). The adjuvants will effectively expose

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the lymphoid tissue to the antigen, with the induction of an immune response as a result.

In the case of intranasal drug delivery, a mucosal immune response is an unwanted effect that should be avoided. However, nasal drug delivery systems such as mucoadhesive gels and microspheres may promote the close contact between an antigen and the epithelial surface needed for activation of the mucosal immune system (O'Hagan and Illum, 1990). Therefore, these experiments were performed to evaluate the immunological implications of nasal drug delivery in dextran microspheres and a thermogelling ethyl(-hydroxyethyl)cellulose (EHEC) system. These nasal drug delivery system have proven successful in promoting insulin absorption in rats (Pereswetoff-Morath and Edman, 1995a,b). To challenge the systems, myoglobin was chosen instead of insulin used in previous studies. Due to its size (17 500 Da) myoglobin is more immunogenic than insulin, but small enough to be incorporated into the nasal delivery systems used. The EHEC system is a complex system that could lose its gelling ability if too large a molecule was incorporated. Also, the myoglobin will be situated on the surface of the G25 spheres, but both on the surface and inside the G50 spheres, similar to insulin in the previous study (Pereswetoff-Morath and Edman, 1995a).

2. Materials and methods

2.1. Materials

Dextran microspheres (Sephadex® G25 and G50) were gifts from Pharmacia, Uppsala, Sweden and ethyl(hydroxyethyl)cellulose (EHEC) was a gift from Berol Nobel, Stenungsund, Sweden. The ELISA plates were Nunc Immunoplate Maxisorp F96. Equine myoglobin and rat IgG were purchased from Sigma Chemicals. Goat-anti-equine myoglobin and HRP-conjugated goat-anti-equine myoglobin were supplied by Bethyl Laboratories Inc., TX, USA. Sheep-anti-rat IgG (γ chain specific), sheep-anti-rat IgA (α chain specific), HRP-conjugated anti-rat IgG (γ chain specific), HRP-conjugated anti-rat IgA (α chain specific), and rat IgA were from The Binding Site, UK. The TMB substrate was from Kirkegaard and Perry Laboratories Inc., MD, USA.

2.2. Preparation of powders and solutions

Dextran microspheres G25 and G50 were sieved and the fraction < 45 μm was mixed with equine myoglobin solutions to yield 40 μg myoglobin per mg spheres. The suspensions were lyophilised and passed through a sieve of 63 μm to yield a homogeneous powder. The amount of myoglobin in the spheres was determined by BCA protein measurement method (Smith et al., 1985). Furthermore, a stock solution of 1% EHEC solution and 3 mM SDS was mixed just before administration with glycerol, *m*-cresol and myoglobin to yield 0.6% EHEC, 1.8 mM SDS, 4.8 mg/ml glycerol, 0.9 mg/ml *m*-cresol, and 2 mg/ml myoglobin as final concentrations.

2.3. Bioavailability of myoglobin

The experiments were essentially performed as described by Rydén and Edman (1992). Equine myoglobin (100 μg per animal) was delivered intranasally in dextran microspheres G25 and G50, in EHEC solution and in physiological saline; 1.25 mg microspheres and 25 μl polymer solution or physiological saline were administered in both nostrils. One group received 10 μg myoglobin per animal i.v. in physiological saline. The analyses of equine myoglobin concentration in the blood were carried out using enzyme-linked immunosorbent assay (ELISA), developed as outlined by Voller et al. (1976).

2.4. Repeated administration of equine myoglobin

Equine myoglobin was administered intranasally to rats in four different delivery systems; dextran microspheres G25, dextran microspheres G50, EHEC solution, and physiological saline. The animals were anaesthetised by inhalation of 4% halothane (Halothane, ISC Chemicals Ltd., UK) for 90 s. The anaesthetic was given in 50% O_2 and 50% N_2O at a flow rate of 1 l/min in a vaporiser specially built for small

animals. The dry microspheres and the solutions were delivered in both nostrils during the 30–45 s that the anaesthesia lasted; 1.25 mg dextran spheres containing 50 μg equine myoglobin were weighed into a PE-90 polyethylene tube and administered by blowing air from a syringe through the tube to each nostril; 25 μl of the solutions were administered to each nostril by a Finnpipett. The intranasal immunisation was performed on days 1, 3, 5, 7, 9, and 11 and a group of three rats received intramuscular injection of equine myoglobin in Freund's complete adjuvant on day 1. All animals received a dose of 100 μg myoglobin per immunisation. A booster injection was given on day 26. The booster contained 100 μg myoglobin in Freund's complete adjuvant for the animals that previously had received intranasal immunisation. Freund's incomplete adjuvant was used for the animals that already had received intramuscular injection of Freund's complete adjuvant on day 1. The negative control animals did not receive any myoglobin.

2.5. Collection of nasal washings and plasma

Blood from the three animals in the i.m. immunised group was drained from the tail vein on days 5, 12, 19, 26, 36, 47, and 79. Blood samples and nasal washings were collected from the animals intranasally immunised on days 5, 12, 19, 26, 36, 47, 65, and 79. On each sampling occasion, three animals in the intranasally immunised groups were anaesthetised with i.p. injection of Inactin® and thereafter blood was taken by cardiac puncture. The blood was centrifuged and the plasma was withdrawn and stored at − 80°C until analysis. The animals received a lethal dose of pentobarbital and nasal washings were collected essentially as described by Kanesaki et al. (1991). The trachea was exposed by a surgical procedure and a fine silk suture was tied around the trachea midway between the larynx and the bifurcation of the trachea. An incision was made and a catheter was inserted towards the nasal cavity. The animals were placed on a tilted support to elevate the feet. Nasal washing was then performed by injection of phosphate buffer (PBS) in the catheter, five times to a total volume of 1 ml. The solution was collected in a tube as it exited the nares. The recovery was approx. 80–90%. Both plasma and nasal washings were analysed for total amounts of IgA and IgG, and for anti-myoglobin IgA and IgG.

2.6. ELISA for immunological response

Two sandwich type ELISAs were developed to determine total IgA and IgG, and specific IgA and IgG against myoglobin, as outlined by Voller et al. (1976). Nunc Immunoplate Maxisorb F96 plates were coated with 100 μl sheep-anti-rat IgA (α-chain specific), sheep-anti-rat IgG (γ-chain specific) or equine myoglobin (20 μg/ml) in 0.05 M sodium carbonate buffer (pH 9.6) with 0.01% thiomersal. The plates were incubated overnight in a moist chamber at 4°C and then washed five times in a Titertec Microplate Washer 120 (Flow Laboratories) with 0.01 M phosphate buffered saline (PBS; pH 7.4) containing 0.1% Tween® 20 and 0.01% thiomersal (PBS-T), and then shaken dry. All subsequent washes were performed in this way. To minimise non-specific binding to the walls of the wells, the plates were incubated with PBS-T for 1 h at room temperature and then shaken dry.

Total IgA and IgG in plasma and nasal washings were determined on plates coated with sheep-anti-rat IgA and sheep-anti-rat IgG; 100 μl of plasma, nasal washings, and rat IgA and rat IgG standards diluted in PBS-T were added to the coated wells. Serial 2-fold dilutions of the samples were performed on the plates. The minimum dilution used was 1:8 to avoid interference from other components in plasma and nasal washings. Following 2 h incubation in a moist chamber at room temperature and subsequent washing, 100 μl HRP-conjugated anti-rat IgA and IgG were added to each well. The second antibodies were diluted 1:160 000 and 1:8000, respectively. After 1 h of incubation in a moist chamber at room temperature the plates were again washed and shaken dry, prior to addition of 100 μl substrate; 3,3′,5,5′-tertramethylbenzidine (TMB). The substrate reaction was terminated after 15 min in room temperature by adding 100 μl 1 M phosphoric acid and the absorbance was then mea-

sured in an microplate reader (Labsystems Multiskan® MCC/340) at 450 nm.

Determination of specific anti-myoglobin antibodies followed almost the same protocol as the measurement of total IgA and IgG. To the equine myoglobin coated plates, 25 μl plasma and nasal washings were added to 175 μl PBS-T in the first well and thereafter serial 2-fold dilutions were performed. Plasma and nasal washings from negative control animals were diluted 1:8 and added to all plates. The incubations and washings were performed as previously described. However, the HRP-conjugated anti-rat IgA was used in a lower dilution, i.e. 1:40 000. Since no standards were available for these measurements, the titres of the samples were determined. The dilution 1:8 in the first well corresponds to the third 2-fold dilution possible to make with an undiluted sample. Therefore, the first well was set to titre 3 and second to titre 4, etc. The highest dilution of the samples that gave an absorbance equal to or greater than the mean absorbance of negative controls plus three standard deviations, was taken as the titre of the sample.

3. Results

3.1. Bioavailability of myoglobin

Myoglobin is rapidly eliminated from the systemic circulation after intravenous injection. A single dose of 10 μg resulted in a plasma concentration of approx. 1 μg/ml after 3 min and after 40 min the concentration was below the lower limit of the assay range (8 ng/ml) of the analytical method used. Myoglobin administered intranasally with dextran microspheres, in EHEC solution, or in physiological saline did not produce plasma levels of myoglobin over the detection limit at any time during the 60 min of the experiments. The largest possible bioavailability of myoglobin after intranasal administration can be estimated using the detection limit as the highest plasma concentration of myoglobin after intranasal administration during this time interval. The calculated bioavailability for myoglobin is less than 1.4%.

3.2. Immunological response

The average amounts of total IgA and IgG in plasma from the different groups were not significantly different from the controls throughout the experiment (Table 1). In nasal washings, the total amounts of antibodies were very low and many of the samples were under the lower limit (25 ng/ml) of the assay range for IgA and IgG. An estimate of the results indicates the total concentration of IgA antibodies to be about 0.3 μg/ml IgA and 0.5 μg/ml IgG in nasal washings in all groups.

Specific IgA antibodies against myoglobin were not detected in plasma nor in nasal washings of any of the test animals. After intramuscular immunisation with equine myoglobin and Freund's complete adjuvant, specific IgG antibodies in the plasma were observed on day 12. Thereafter the titre continuously increased until a plateau at titre 13 was reached on day 47. The intranasal immunisation did not generate an IgG response, but when the animals were boosted i.m. with Freund's complete adjuvant on day 26 they produced an IgG response identical to the primary response observed in the i.m. group (Fig. 1).

4. Discussion

Intranasal administration of myoglobin in physiological saline or together with absorption promoting systems like dextran microspheres and

Table 1
Total IgA and IgG in plasma during the experiment

Administration route	Delivery system	Antibody concentration in plasma (μg/ml)	
		IgA (S.D.)	IgG (S.D.)
-	Negative control	6.81 (0.91)	517.7 (127.8)
i.n.	Microspheres G25	6.44 (1.28)	729.4 (190.4)
i.n.	Microspheres G50	6.16 (1.03)	696.5 (191.4)
i.n.	EHEC	5.98 (0.94)	733.1 (208.9)
i.n.	Phys. saline	5.87 (1.07)	728.1 (200.3)
i.m.	Phys. saline	6.47 (1.07)	677.9 (170.6)

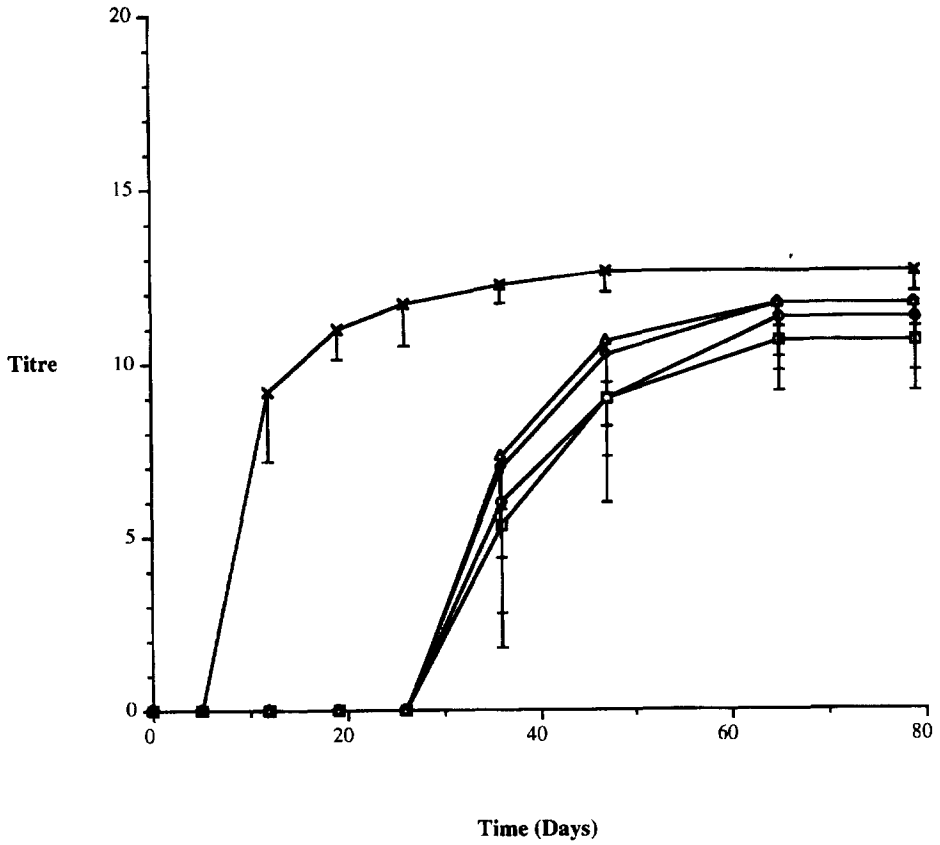

Fig. 1. Titres of specific IgG against equine myoglobin (mean ± S.D.) after (x) intramuscular injection of 100 μg equine myoglobin with Freund's complete adjuvant on day 1 and intranasal immunisation of 100 μg equine myoglobin on days 1, 3, 5, 7, 9, and 11 in (□) dextran microspheres G25, (◇) dextran microspheres G50, (○) EHEC, and (△) physiological saline. All groups received an intramuscular booster dose of 100 μg equine myoglobin on day 26.

EHEC show a very low bioavailability. No detectable amounts of equine myoglobin enter the systemic circulation after nasal administration. The lack of systemic immune response after intranasal administration is in congruence with these findings. The i.m. booster dose showed that the animals were immunocompetent and that the lack of response was not due to a deficient immune system in these animals. The booster also revealed that the animals reacted with a primary response similar to the response after i.m. immunisation. In a secondary response, as a result of priming of the immune system by intranasal immunisation, the specific IgG antibodies should have been produced more rapidly than was observed. This is evidence that the intranasal administration has not sensitised the systemic immune system to equine myoglobin. There could be several explanations to these findings. First, myoglobin is a weak antigen not able to induce an immunological response in low systemic concentrations, which would be the case after nasal administration. Second, the pharmacokinetic profile of myoglobin shows rapid elimination from the circulation and consequently the time for exposure to the immunocompetent cells is short. Intramuscular injection with Freund's adjuvant acts as a depot and myoglobin is released during an extended period of time, hence the equine myoglobin is presented to the immune system in an optimal manner.

A mucosal response should have produced an increase in anti-myoglobin IgA antibodies in the nasal washings since this is the predominant isotype of antibodies in the mucosal immune system. However, no specific IgA antibodies were found in the washings in any of the intranasally administered animals. Part of the explanation might be the low irritable and inflammatory properties of the delivery systems (Pereswetoff-Morath et al., 1996). Inflammation or irritation attracts immunocompetent cells that would promote a local immunological response.

In summary, dextran microspheres and EHEC do not act as adjuvants, potentiating the immunogenicity of foreign proteins. This is an important property for a nasal drug delivery system to fulfil and necessary for the long-term use of systems that should improve or optimise the absorption of proteins or peptides when given nasally.

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