

Starch microparticles as an adjuvant in immunisation: effect of route of administration on the immune response in mice

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Abstract

This paper describes the effects on the development of an immune response by changing the route of administration of a new vaccine adjuvant, starch microparticles with human serum albumin (HSA) as a model antigen. The model vaccine was administered to mice by oral, subcutaneous and intramuscular routes in various combinations and both the local secretory immunoglobulin antibody (s-IgA) and systemic humoral and cellular (delayed-type hypersensitivity assay (DTH)) responses were followed. The only immunisation regimens inducing a significant s-IgA response were those incorporating oral booster doses. Oral and subcutaneous immunisations had similar effects on the Th1/Th2 balance, as indicated by the IgG subclass ratios and cytokine analyses. However, significant differences between oral and intramuscular immunisations were seen in the IgG subclass ratios. The Th2 influence was stronger after oral primary immunisation than after intramuscular primary immunisation, while oral boosters elicited a comparatively stronger Th1 response than intramuscular boosters. This result was also supported by the DTH analyses. Subcutaneous immunisation induced a stronger Th2 response than intramuscular immunisation, as indicated by subclass ratio and the IgE response. In conclusion, our results show that the profile of an immune response depends on the route of administration, which should be considered when developing new vaccines or new routes of administration.

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1. Introduction

Detailed information on the specific components of an immune response that are essential for protection during an infection is normally not yet available. However, it is well known that the effects of some toxins, such as those from *Corynebacterium diphtheria* or *Clostridium tetani*, are effectively neutralised by the specific humoral responses induced by vaccination, and that a Th1 cellular response is effective in intracellular infections caused by bacteria such as *Bordella pertussis* [1] or *Mycobacterium tuberculosis* [2,3]. The significance of a mucosal response or a change in the Th1/Th2 balance in some infections is not completely understood but it is reasonable to speculate that the importance of the different components varies with the infection. It is therefore important, when designing the optimal vaccination

strategy for a vaccine, to know how this differentiation of the immune response is affected by the choice of adjuvant, the route of administration and the dose or number of doses.

Protection from infection is also often associated with a mucosal immune response, i.e. the production of secretory immunoglobulin antibodies (s-IgA), as the vast majority of pathogens initially infect the host via the mucosal membranes. Usually, the induction of peripheral immune responses by parenteral immunisation does not result in significant mucosal immunity. Mucosal immunisation is considered necessary for induction of a mucosal immune response, whereas it is possible to induce a systemic immune response by either parenteral or mucosal immunisation [4,5], although a combination of mucosal and parenteral approaches generates a stronger immune response than either route alone [6–8].

Our laboratory has developed a delivery system for an oral vaccine adjuvant composed of starch microparticles. Human serum albumin (HSA) was conjugated to starch microparticles for use as a model antigen in vaccination studies. The HSA-microparticles induce strong local and systemic immune responses in mice [9]. In particular, a

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strong cellular response, detected by a delayed-type hypersensitivity (DTH) test, and a strong mucosal IgA response were induced. A similar response and protection from infection were seen when secreted antigens from *Salmonella enteritidis* were conjugated to the starch microparticles and given orally to mice [10].

In this study, we used HSA as a model antigen conjugated to the microparticle carrier system, and characterised the immune response after oral and parenteral (subcutaneous or intramuscular) administration in different immunisation schemes, where the route of administration was changed after the primary immunisation or after the first booster dose.

2. Materials and methods

2.1. Preparation of polyacryl starch microparticles

The microparticles were prepared by polymerisation of acryloylated starch (maltodextrin, MD6, Stalex AB, Malmö, Sweden) in a w/o-emulsion, as previously described [11,12]. Briefly, acryloylated starch (500 mg) was dissolved in 5 ml of a 0.2 M sodium phosphate buffer, at pH 7.5, containing 1 mM EDTA. Ammonium peroxodisulfate was added to give a final concentration of 0.8 M in the aqueous phase, which was homogenised in 300 ml toluene:chloroform (4:1) with 0.08 g Pluronic® F68 as an emulsifier. *N,N,N',N'*-tetramethylethylenediamine (TEMED) (Sigma, St. Louis, MO, USA) was used to initiate the polymerisation.

2.2. Conjugation of human serum albumin to polyacryl starch microparticles

Human serum albumin (HSA, Aventis Behring, Germany) was conjugated to the microparticles using the carbodiimide method of Bethell et al. [13]. Microparticles were activated with carbonyldiimidazole (CDI, Merck, Darmstadt, Germany) in dimethylformamide (DMF) during rotation for 1 h at room temperature. After several centrifugal washings with DMF to remove unreacted CDI, the microparticles were transferred through a washing step into saline solution before being resuspended into the coupling buffer, 0.25 M boric acid with 0.15 M NaCl at pH 8.6, containing HSA. The vessel containing the mixture was rotated at 4 °C for 72 h end-over-end. The microparticles were then washed several times with saline, filtered through an 11 µm nylon filter, and stored at 4 °C. The amount of HSA conjugated to the microparticles was determined by amino acid analysis. The amount of microparticles per millilitre was determined by dry weight. The size-number distribution of the microparticles was determined using a laser diffractometer (LS 230, Coulter, USA). The mean (±S.D.) diameter of the various batches of microparticles used in these studies was 2.0 ± 0.35 µm, based on the number distribution.

2.3. Animals

The female inbred BALB/c mice Abom strain (Bomholtgård, Ry, Denmark), 12–14 weeks old, used in these experiments were kept according to Good Laboratory Practice guidelines under the supervision of a licensed veterinarian. Water and a standard pelleted diet were available ad libitum. The regional Animal Ethics Committee, Uppsala, approved the experiments.

2.4. Immunisations

The immune response was investigated by measuring the antibody titres IgM + IgG and the subclasses IgG1, IgG2a, IgG2b in serum, the specific secretory IgA content in faeces, cytokine production of primed, in vitro stimulated splenocytes (experiments 1 and 3) and the cellular immune response in vivo, using a DTH test (experiment 2); it was not possible to perform both the latter analyses in the same animals. The immune response was then compared in groups receiving immunisation by two different routes per regimen and in relevant control groups receiving the HSA-microparticles either solely orally or parenterally. In detail, three sets of immunisation studies were performed, both following the specific humoral responses to HSA in blood and faeces. The Th1/Th2 balance, including analysis of the cytokines released after activation of primed spleen cells, was studied in experiment 1 and 3 (Fig. 1a and c). The cellular response was studied in a different group of mice in experiment 2 (Fig. 1b).

In experiment 1, BALB/c mice (six per group) received a primary immunisation and three booster doses. The three groups of mice received the total immunisation course subcutaneously, orally, or with a change of administration route from subcutaneous to oral between the first and second booster, as shown in Fig. 1a. The subcutaneous groups received 1 mg of HSA-microparticles in 0.1 ml physiological saline in the scruff of the neck. The oral groups received 3 mg of HSA-microparticles in 0.2 ml physiological saline on three consecutive days by gastric intubation. The microparticles contained 30 µg HSA/mg in the first two doses and 40 µg HSA/mg in the last two doses.

In experiment 2, eight BALB/c mice per group were immunised orally, intramuscularly or with a change of administration route from oral to intramuscular or vice versa (Fig. 1b). The intramuscular immunisations were given as an injection of 1 mg HSA-microparticles in 0.1 ml physiological saline in the hind leg and the oral immunisations were given as described above. The microparticles used in experiment 2 contained 97 µg HSA/mg.

Since the subcutaneous route was used in experiment 1 and the intramuscular route was used in experiment 2, a third experiment (Fig. 1c) was performed in order to compare these parenteral routes. In experiment 3, six

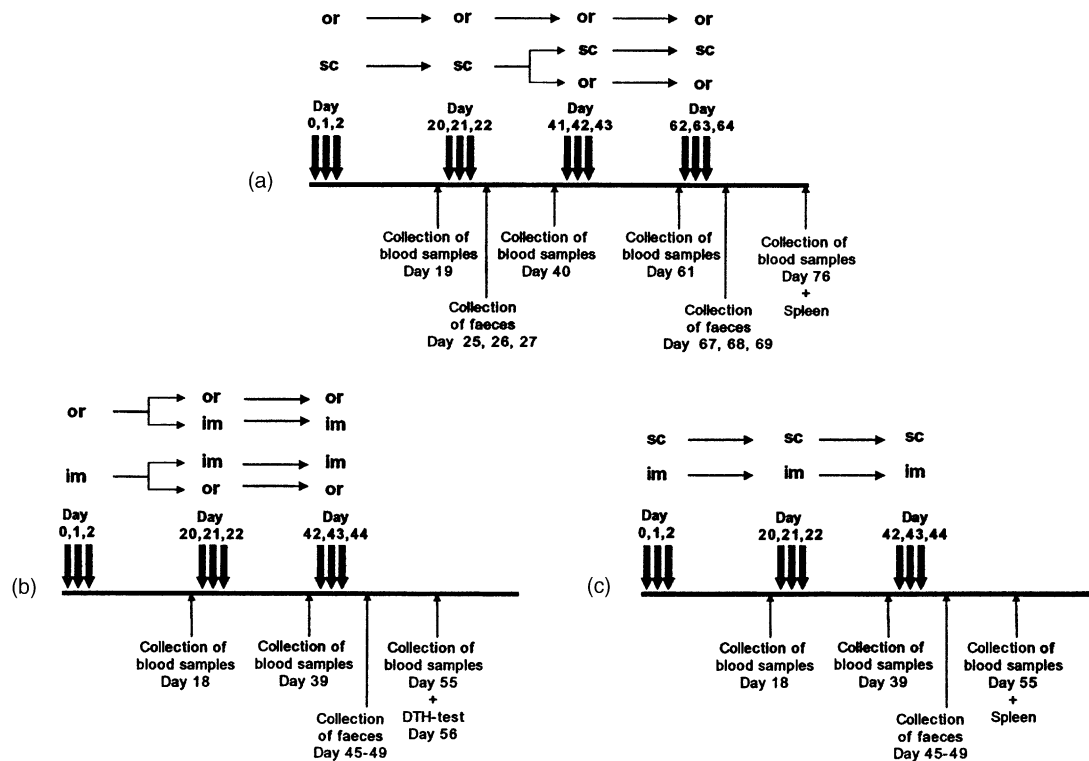


Fig. 1. Immunisation protocols for experiments 1–3 (a–c). Thick arrows show oral immunisations in series of 3 consecutive days; parenteral immunisations (im or sc) were given on the first day in each respective series. For further information, see text in Section 2.4.

BALB/c mice per group were immunised subcutaneously in the scruff of the neck or intramuscularly in the hind leg. Each immunisation was given as an injection of 1 mg HSA-microparticles in 0.05 ml physiological saline. The microparticles used in experiment 3 contained 44 μ g HSA/mg. The HSA-specific immune response was studied as in experiment 1.

The boosters in all three experiments were given 3 weeks apart.

2.5. Collection and preparation of blood and faecal samples

Blood samples were taken from the tail artery. Serum was prepared by centrifugation and stored at -20°C . Six to ten samples of freshly voided faeces were collected into Erlenmeyer tubes before and intermittently after each immunisation dose, frozen and freeze dried. After the net dry weights were determined, extracts of the faeces were prepared, keeping all samples on ice. Briefly, phosphate-buffered saline (PBS), containing 5% non-fat dry milk, 0.1 mg soybean trypsin inhibitor per millilitre (Sigma) and 2 mM phenylmethylsulphonylfluoride (Sigma), was added at a concentration of 20 μ l solution per milligram of dry faeces. Solid matter was suspended by rigorous vortexing and separated by centrifugation at $13,000 \times g$ for 10 min at 4°C . The clear supernatants were frozen at -20°C until analysed for antigen-specific and total IgA.

2.6. Splenocyte cultures

Immunised and naïve mice were sacrificed 2 weeks after the last booster and the spleens were removed aseptically. The spleens were pooled by group and punctured with a thin syringe. The cells were then squeezed out to prepare single-cell suspensions, washed in sterile PBS, and incubated on ice for 10 min in Tris buffer with ammonium chloride. After one wash in RPMI 1640 medium, the cells were resuspended in culture medium (RPMI 1640 containing 5% foetal calf serum (FCS)), 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ M 2-mercaptoethanol and 2 mM L-glutamine (Gibco BRL, Gaithersburg, MD), and were then counted. They were plated (5×10^6 per well, with a total volume of 1.5 ml per well) into sterile 24-well microplates (Costar, Corning Inc., NY) and stimulated either with HSA (1, 0.1 and 0.01 mg/ml in the wells), concanavalin A (Con A, 4 μ g per well) (Sigma) or, as a negative control, with sterile RPMI-medium. Cultures were incubated for 48 and 72 h at 37°C in 5% CO_2 . The cells were harvested by centrifugation at $5000 \times g$ for 10 min at 4°C and the supernatants were collected and frozen at -20°C until analysed for cytokine production.

2.7. Delayed-type hypersensitivity test

One week after the last immunisation, DTH tests were performed by measuring the increase in ear thickness 24,

48 and 72 h after an intradermal challenge in the left ear (ear A) with 10 μ l of 1 mg/ml HSA in physiological saline [9]. As a negative, internal control for each mouse, 10 μ l of physiological saline was injected into the right ear (ear B). The relative increase in ear thickness (at time t) was calculated from the formula $[(A_t - A_0) - (B_t - B_0)] \times 100/A_0$.

2.8. Assessment of antigen-specific antibodies

Nunc Immunoplate Maxisorb F96 plates were coated with HSA (12.5 μ g per well) in 0.05 M sodium bicarbonate buffer (pH 9.6) containing 0.05% NaN_3 for 2 h in a moist chamber at room temperature. The plates were washed in 0.01 M PBS (pH 7.4) with 0.05% Tween 20, shaken dry and stored in a desiccator at 4 °C until further use. Before use, the plates were incubated with PBS with 0.2% Tween 20 for 1 h at room temperature to avoid nonspecific binding. The antisera or extracts of faeces were diluted to appropriate concentrations in PBS with 0.2% Tween 20 and added to the plates in series of two-fold dilutions. The following antibodies were used as secondary antibodies: alkaline phosphatase-conjugated goat-anti-mouse IgM + IgG (Biosource, CA, USA), goat-anti-mouse IgA (Sigma), rat-anti-mouse IgG2a, rat-anti-mouse IgG2b and rat-anti-IgG1 (Pharmingen, San Diego, CA, USA). The substrate was 4-nitrophenylphosphate (Merck) 1 mg/ml in 10% diethanolamine buffer, pH 9.6, with 0.5 mM MgCl_2 . The absorbance was measured at 405 nm in a microtitreplate spectrophotometer (Titertek Multiscan MC, Flow Laboratories).

For IgE determination, the HSA-coated plates were incubated with 10% FCS in PBS for 1 h to avoid unspecific binding. The antisera were diluted to appropriate concentrations in 10% FCS in PBS and added to the plates in series of two-fold dilutions. A mixture of biotin-conjugated rat anti-mouse IgE (1:250) and avidin-HRP (Pharmingen, San Diego, CA, USA) (1:250) was used for detection. Tetramethylbenzidine (Sigma) 0.1 mg/ml in 0.1 M NaAc buffer, pH 6.0 with 0.2% H_2O_2 was used as substrate. The reaction was stopped with stop solution, 2 M H_2SO_4 . The absorbance was measured at 450 nm.

A sandwich-type enzyme-linked immunosorbent assay (ELISA) was developed to determine IgG1, IgG2a and IgG2b in sera against relevant subclass standard curves. Nunc Immunoplate Maxisorb F96 plates were coated with rat-anti-mouse IgG1, IgG2a or IgG2b (Pharmingen) and incubated overnight in a moist chamber at 4 °C. The plates were blocked with 1% OVA and the standard mouse IgG1, IgG2a or IgG2b was diluted to appropriate concentrations in PBS with 3% OVA to create standard curves. The subsequent procedures were performed as described above. In each sample, the final concentration of antigen-specific IgG1, IgG2a or IgG2b was determined from pooled standard curves by calculating the mean concentration from three dilutions of a sample.

When measuring IgM + IgG antibodies and subclasses in serum, pooled negative serum was added to each plate as a negative control. The negative serum absorbance values from all the plates were below 0.1. Thus, 0.1 was set as the limiting value for a positive result. When measuring IgE antibodies in serum, the negative serum absorbance value of that specific plate was used as the limiting value for a positive result. Titres are given as $-\log_2$ (dilution $\times$ 10). A positive sample (from hyperimmunised mice) was included as a standard on all plates and treated in the same way as the antisera to ensure consistency between plates.

2.9. Assessment of total IgA in faecal samples

A sandwich-type ELISA was developed to determine the total amount of IgA in faecal samples. Plates were coated with goat-anti-mouse IgA (0.5 μ g per well) (Sigma) overnight in a moist chamber at 4 °C. The plates were blocked with 1% OVA and the standard IgA (Sigma) was diluted to appropriate concentrations in PBS with 1% OVA to create a standard curve. The extracts from the faecal samples were diluted in PBS with 0.2% Tween 20 and added to the plates in series of two-fold dilutions. The concentrations of specific and total IgA were determined from a pooled standard curve by calculating the mean concentration from three dilutions of the sample. The standard curves were linear in the range of 1–60 ng/ml. Positive and negative samples were included on all plates and treated in the same manner as the unknown samples. The negative faeces absorbance values from all plates were below 0.1. Thus, 0.1 was set as the limiting value for a positive result. An absorbance of 0.1 corresponded to 8 ng/ml. The final concentrations of total and specific IgA were determined from pooled standard curves by calculating the mean concentration from three dilutions of a sample.

2.10. Assessment of cytokines in supernatants from cultured splenocytes

A sandwich-type ELISA was developed to determine the amount of interferon (IFN)- γ , interleukin (IL)-2, IL-4 and IL-5 produced by the splenocytes during antigen activation. Antibody pairs and recombinant standards were purchased from Pharmingen. Nunc Immunoplate Maxisorb F96 plates were coated in 0.1 M sodium bicarbonate buffer at pH 9.6 with rat-anti-mouse IFN- γ , IL-2, IL-4 or IL-5, and incubated overnight in a moist chamber at 4 °C. The plates were blocked with 1% OVA. The standards (IFN- γ , IL-2, IL-4 or IL-5) were diluted to appropriate concentrations in PBS with 1% OVA to create appropriate standard curves. The supernatants from cell media were added in duplicate to the plates in series of two-fold dilutions. Biotin-conjugated rat-anti-mouse IFN- γ , IL-2, IL-4 or IL-5, diluted in PBS with 1% OVA, was added to the plates, followed by avidin-peroxidase and, later, 2,2'-azino-bis-3-ethylbenzthiazoline-sulfonic acid (ABTS,

Sigma) with 0.3% H₂O₂. The reaction was stopped with 0.7 M SDS in DMF and purified water, 1:1 (Sigma). The absorbance was measured at 405 nm. In each sample, the final concentration of IFN- γ , IL-2, IL-4 or IL-5 was determined from the standard curves by calculating the mean concentration from three dilutions.

2.11. Statistics

Data analysis was performed in part using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego, CA, USA, <http://www.graphpad.com>. The significance of difference between two groups was tested using the t-test for unpaired samples. The one-way analysis of variance (one-way ANOVA) with Newman–Keuls Multiple Comparison Test was used to test significant differences among more than two groups. A two-way ANOVA was performed on the subclass ratio in experiment 2 to analyse significances of the route of administration on primary and booster doses. The two-way ANOVA was also used to analyse the significance of difference between administration route in experiment 3. The repeated-measures analysis of variance in conjugation with Fisher's multiple comparison test was performed, using the Software Statview 4.0, to analyse the IgA quota and the DTH response. Data from the subclass ratios in serum were logarithmically transformed. Arithmetic data were used for the IgM + IgG and IgE titres in serum, the IgA quota in faeces and the DTH results. A *P*-value of less than 0.05 was considered to be significant.

3. Results

3.1. HSA-microparticles induce a strong humoral response independently of administration route

As seen in Fig. 2 (experiment 1), there was a strong systemic anti-HSA response by day 76 in all groups af-

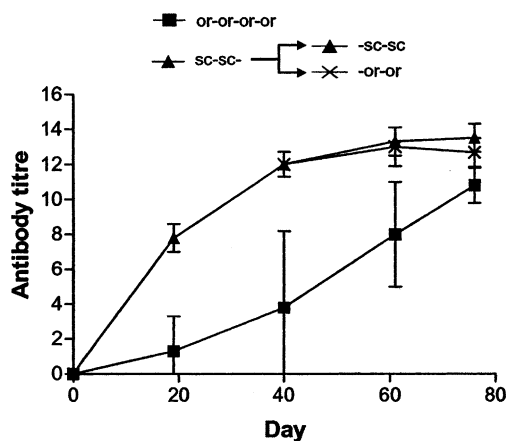


Fig. 2. Serum HSA-specific IgM + IgG responses after immunisation with HSA-microparticles according to Fig. 1a. Mean antibody titres are given as $-\log_2$ (dilution $\times 10$) ($\pm$ S.D.) ($n = 12$ or 6).

ter three booster doses. There were no differences in the IgM + IgG response between the groups receiving at least some doses subcutaneously (i.e. sc-sc-or-or and sc-sc-sc-sc). The antibody response in the group that only received oral doses (or-or-or-or) was slower in onset than that in the other groups. Only three of the six mice in the oral group had responded after two booster doses (day 40). However, all six mice had responded by day 76, after three booster doses, with an IgM + IgG antibody titre approaching those of the mice primed subcutaneously. In experiment 2, where the parenteral immunisation was given intramuscularly, there were no significant differences in IgM + IgG antibody titres between the groups, with group mean titres ranging from 11.4 to 12 at day 55. In experiment 3, where the immunisations were intramuscular or subcutaneous (i.e. im-im-im or sc-sc-sc), the IgM + IgG response was also high, with endpoint titres of 13.7 ± 0.5 and 13.2 ± 0.4 , respectively.

3.2. A mixed Th1/Th2 response, which is influenced by the route of administration

The subclass profile of the humoral IgG response, i.e. antigen-specific IgG1, IgG2a and IgG2b, was analysed using ELISA in order to follow the Th1/Th2 profile. All three subclasses were detected, indicating a mixed Th1/Th2 response, and the concentration of each in serum increased over time, with IgG1 dominating. The results from experiment 1 are shown in Fig. 3. The ratio of antigen-specific IgG1 to the sum of antigen-specific IgG2a and IgG2b (as a measure of the Th1/Th2 profile) was calculated for each individual mouse; the mean group results from all the

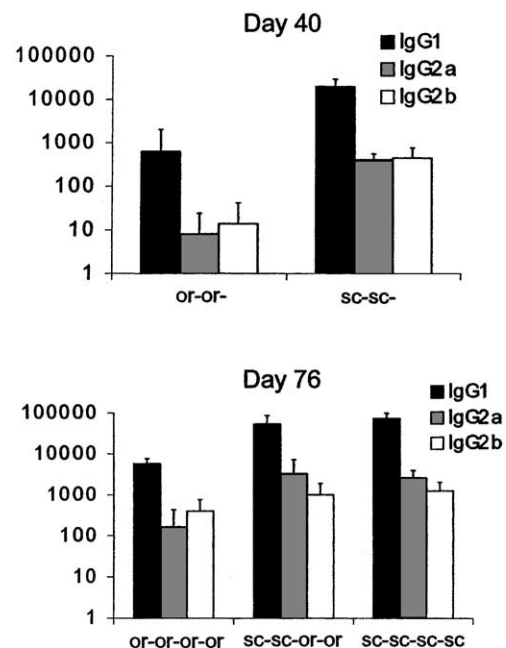


Fig. 3. HSA-specific subclass profile in serum after the first (day 40, $n = 3$ –6 or 12) and third booster (day 76 $n = 6$) according to Fig. 1a. The bars represent means in $\mu\text{g/ml}$ ($\pm$ S.E.M.).

Table 1

The subclass ratio in serum after immunisation with HSA-microparticles according to Fig. 1a–c

Experiment	Immunisation	Mean subclass ratio $\pm$ S.E.M. ^a		
		Day 40	Day 55	Day 76
1	or-or-or-or	22.4 ^b $\pm$ 7.1 (n = 3)		25.1 $\pm$ 9.9 (n = 6)
	sc-sc-sc-sc	25.5 $\pm$ 3.7 (n = 12)		16.0 ^c $\pm$ 2.3 (n = 5)
	sc-sc-or-or			18.6 $\pm$ 5.0 (n = 6)
2	im-im-im		82.2 $\pm$ 23.2 (n = 6)	
	im-or-or		24.8 $\pm$ 4.0 (n = 8)	
	or-or-or		147.6 ^e $\pm$ 48.1 (n = 7)	
	or-im-im		480 8 ^d $\pm$ 213.3 (n = 7)	
3	im-im-im		7.7 $\pm$ 2.0 (n = 6)	
	sc-sc-sc		56.2 $\pm$ 18.3 (n = 6)	

^a Values represent arithmetic means ($\pm$ S.E.M) of serum HSA-specific ratios of IgG1/(IgG2a + IgG2b) after the first (day 40) and last (days 55 and 76, respectively) boosters.

^b Only responding mice are included.

^c One value was found to be an outlier and was excluded.

^d The ratio was significantly higher in the or-or-or group than in the im-or-or group ($P < 0.05$). Primary oral immunisation resulted in a higher ratio ($P < 0.0001$) than primary intramuscular immunisation, while the ratio was lower with an oral booster ($P < 0.05$) than with an intramuscular booster. Statistical analyses were performed using logarithmically transformed values; for details see Section 2.

^e The ratio was significantly higher in the or-im-im group than in the im-im-im ($P < 0.05$) or im-or-or ($P < 0.001$) groups.

experiments with different immunisation regimens are presented in Table 1. The results between the different routes of administrations were compared within each experiment.

No significant differences between the subclass ratios were seen in experiment 1 (using the oral and subcutaneous routes of administration). However, in experiments 2 and 3, some differences were detected. Thus, in experiment 2, the ratio was higher with primary oral immunisations than with primary intramuscular immunisations ($P < 0.0001$; two-way analysis of variance) indicating that, by the end of the immunisation programme, the oral primary immunisation had a higher Th2 influence than intramuscular primary immunisation. However, the ratios were significantly lower in mice receiving oral boosters than in those receiving intramuscular boosters ($P < 0.05$), indicating that oral booster doses increase the Th1 influence compared to intramuscular booster doses. Experiment 3 confirmed the results obtained from the earlier experiments showing no differences between oral and subcutaneous routes and oral primary vaccination resulting in greater Th2 influence, in the sense that the subcutaneous route resulted in higher ratios (higher Th2 influence) than the intramuscular route at the end of the vaccination programme ($P < 0.0001$).

The specific IgE response was detected in all immunised mice, confirming the influence of a Th2-type response (Fig. 4). The response also reflects the previously reported IgM + IgG response, with increasing titres over time and a slower onset in the mice immunised orally in experiment 1 (Fig. 4a). In experiment 2, the IgE response was the same in all the groups at all time points (Fig. 4b). However, in experiment 3, the group immunised subcutaneously had a significantly higher IgE response ($P < 0.001$) than the intramuscular group (Fig. 4c), confirming the higher Th2 influence detected earlier.

3.3. Oral but not parenteral boosters with HSA-microparticles induce a strong mucosal immune response

The specific s-IgA content in faeces obtained in experiment 1 is shown for days 67–69 (the third to fifth day after the third booster) in Fig. 5. Only the mice that received the oral booster doses with HSA-microparticles (or-or-or-or and sc-sc-or-or) showed an s-IgA response, and the mice that received only oral immunisation had the highest s-IgA response. Mice receiving only subcutaneous immunisation had a very low s-IgA response in faeces, which did not increase over time. The s-IgA response on days 25–27 (the third to the fifth day after the first booster) was low, with the highest response (approximately 0.3 μ g/mg of total IgA) detected in mice that received oral immunisations.

Similar results were seen in experiment 2, in which the s-IgA response increased with time in the mice that received oral booster doses, i.e. or-or-or and im-or-or. In contrast, intramuscular immunisation with the microparticles did not induce any detectable s-IgA, even in the mice receiving an oral primary immunisation followed by two intramuscular immunisations (or-im-im; data not shown). However, in experiment 3, a very low specific s-IgA response was detected in both the subcutaneous (approximately 0.04 μ g/mg of total IgA) and the intramuscular (approximately 0.13 μ g/mg of total IgA) groups.

3.4. Primed splenocytes produce high levels of IFN- γ , IL-2 IL-5 and some IL-4 after in vitro stimulation with HSA

Splenocytes from immunised (experiment 1 and 3) or naïve mice were pooled and restimulated in vitro with HSA 2 weeks after the last booster. The supernatants were

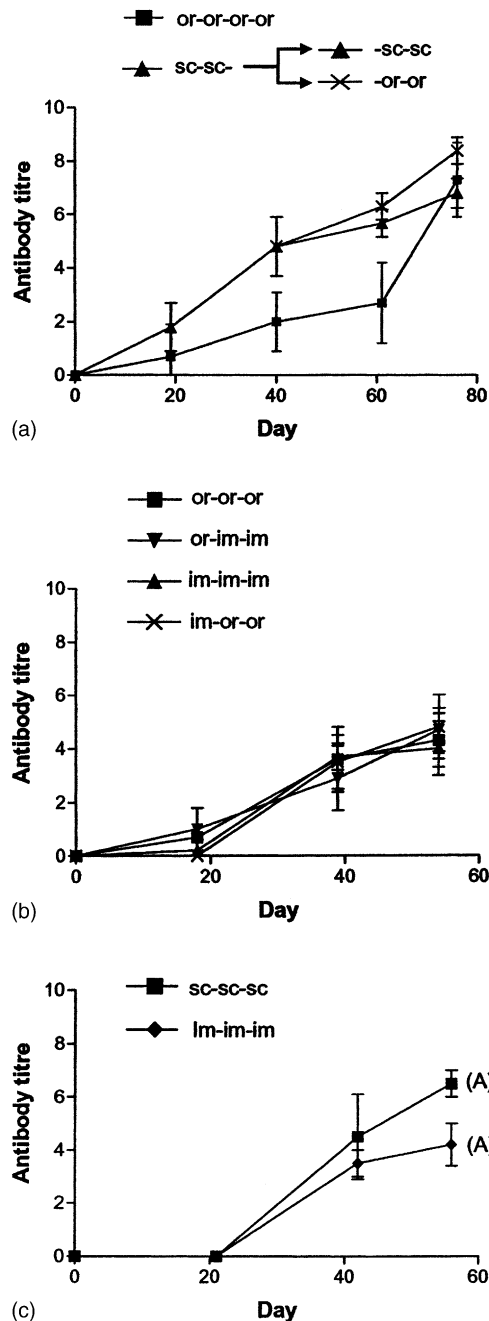


Fig. 4. (a–c) HSA-specific IgE response in serum after immunisation with HSA-microparticles according to Fig. 1a. ($n = 5–6$), Fig. 1b. ($n = 5–7$) and Fig. 1c. ($n = 6$), respectively. Mean antibody titres are given as $-\log_2$ (dilution $\times 10$) ($\pm$ S.D.). (A) Indicates that the values are significantly different, $P < 0.001$ (two-way ANOVA).

collected on days 2 and 3 and assayed in duplicate for IFN- γ and IL-2, representing a Th1 response, and for IL-5 and IL-4, representing a Th2 response. The response to HSA was linked with the dose; cytokine production increased with increased concentrations of HSA. Further, production of all the cytokines except IL-2, which peaked on day 2, increased from days 2 to 3. All three groups immunised with HSA-microparticles in experiment 1 responded with high

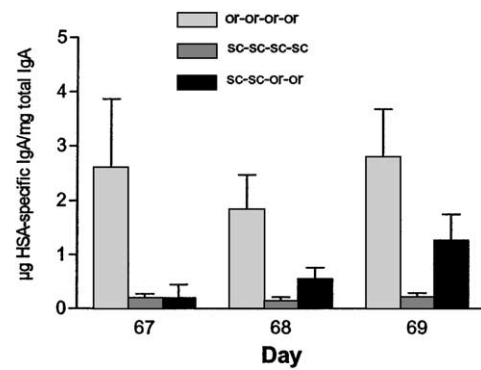


Fig. 5. HSA-specific IgA in faeces after the third booster according to Fig. 1a. Values are given as means in $\mu\text{g/ml}$ total IgA ($\pm$ S.E.M.) ($n = 6$). The group immunised orally (or-or-or-or) responded with significantly higher quotas than the other groups over time ($P = 0.0014$ and <0.0001 , respectively).

amounts of IFN- γ , IL-2 and IL-5 on restimulation with HSA, indicating a mixed Th1/Th2 response. No IL-4 response was detected in any of the groups at the time points assayed. In experiment 3, both immunised groups responded with IFN- γ , IL-2, IL-5 production and also with low amounts of IL-4 (Table 2). The IL-2 response was somewhat lower in experiment 3 than in experiment 1. Taken together, the *in vitro* production of cytokines by stimulated spleen cells supports the previous results, indicating that a mixed Th1/Th2 response was induced in the immunised mice.

3.5. Induction of a high cellular immune response in all immunised mice by the oral and intramuscular route of administration or a combination thereof

A DTH test was performed to study the development of the cellular immune response. The mice immunised in experiment 2 were challenged intradermally in the ear 2 weeks after the last (second) booster and the increase in ear thickness was measured at 24, 48 and 72 h. A strong DTH (cellular) response was seen after 72 h in all the groups immunised with the HSA-microparticles, independently of

Table 2
Cytokine analyses in supernatants from activated primed splenocytes^a after 48 or 72 h

Experiment	Immunisation ^a	IFN- γ (ng/ml)	IL-2 (ng/ml)	IL-5 (ng/ml)	IL-4 (ng/ml)
1	or-or-or-or	3.3	1.2	3.0	ND
	sc-sc-sc-sc	3.1	0.8	2.3	ND
	sc-sc-or-or	2.9	1.2	2.2	ND
3	im-im-im	3.2	0.7	4.6	0.2
	sc-sc-sc	3.2	0.8	3.4	0.1
	Naïve	ND	0.2	ND	ND

^a Mice were immunised according to Fig. 1a or c and sacrificed 2 weeks after the last booster. Splenocytes were pooled by group and stimulated *in vitro* with 1 mg of HSA per well. Cytokine assays were performed on duplicate supernatants and analysed 48 h (IL-2) or 72 h (IFN- γ , IL-5 and IL-4) after stimulation. ND: not detected.

Table 3
The delayed-type hypersensitivity (DTH) response 72 h after intradermal challenge

Group ^a	Percentage increase in ear thickness 72 h after challenge (mean $\pm$ S.D.) ^b
or-or-or	80 $\pm$ 37 (<i>n</i> = 4)
or-im-im	62 $\pm$ 12 (<i>n</i> = 4)
im-im-im	78 $\pm$ 21 (<i>n</i> = 3)
im-or-or	115 $\pm$ 68 (<i>n</i> = 3)
Naïve	3 $\pm$ 7 (<i>n</i> = 3)

The DTH response was measured as the increase in ear thickness after injection of 10 μ l HSA antigen in the ear 2 weeks after the second booster.

^a Groups of mice were immunised according to Fig. 1b.

^b Values are given as arithmetic means ($\pm$ S.D.). The increase in ear thickness was significantly greater in all groups immunised with HSA-microparticles than in the naïve mice (*P* < 0.01).

administration route (Table 3). However, there was a trend for the cellular response to be higher in the mice boosted orally. This also supports the previous results that oral boosters lowered the subclass ratio, indicating a higher Th1 influence after oral boosters than after intramuscular boosters.

4. Discussion

It is important, when introducing a new adjuvant or route of administration for a vaccine, to consider the potential alteration of the character of the immune response. Experience gained to date indicates that a specific immune response profile, dependent on the specific infection/antigen, is required for protection. A key factor in this respect appears to be the site at which the vaccine/antigens are introduced, as different local cytokine concentrations can regulate the Th1/Th2 balance differently. Since this balance subsequently decides the differentiation of the immune response, it is essential that the character of the response to any new adjuvant is known, so that its addition will not adversely change an immune profile which has previously been shown to be effective.

Although we do not yet have detailed knowledge of the consequences of changing routes of administration on the profile of the immune response, several reports confirm that using different routes for the primary and booster vaccinations can synergistically broaden and quantitatively improve the immune response [6–8,14]. Thus, e.g. McNeela et al. [8] showed that a nasal booster after an intraperitoneal primary vaccination with diphtheria CRM₁₉₇ and chitosan enhanced the specific serum IgG and mucosal s-IgA responses. Similarly, Chattaraj et al. [7] reported that specific IgG was highest when an influenza vaccine was given both subcutaneously and orally. Recently, John et al. [14], using different cholera toxin preparations, showed that a combination of transcutaneous and oral administration provided the strongest response. These findings are important for such considerations as the need to improve herd immunity to, e.g. diphtheria using an oral booster after primary parenteral vaccination of newborn children. However, it is important to

emphasise that it is not advisable to generalise; the properties of each adjuvant and antigen/infection must be characterised to ascertain that, for example, an oral booster dose really does give the expected improvement in immune response.

Despite the experimental variations seen in our studies, some concrete conclusions can be drawn. Thus, the most obvious difference between the dosage regimens studied was the development of a strong mucosal response after oral immunisation. Only low amounts of s-IgA were detected after subcutaneous or intramuscular administration. Parenteral primary vaccination followed by oral boosters had no synergistic effect on the s-IgA profile. Nor did we detect any increased effect on the IgM + IgG titres when parenteral and oral vaccinations were combined, which was surprising considering the findings of other groups [6–8]. The final titres were essentially the same in all dosage regimens, which may have been because the starch microparticles were so effective as an adjuvant that the titres reached the maximal plateau values.

The cellular response, as estimated by the DTH assay at the end of the immunisation programme, suggests that oral administration of the model vaccine elicits a higher inflammatory reaction than intramuscular immunisation, which indicates a higher Th1 influence [15]. The higher Th1 influence was also detected in the subclass analyses, which showed that oral boosters after oral or intramuscular primary immunisation induced a lower IgG1/IgG2_{a+b} ratio than boosters given intramuscularly. Thus, our results using starch microparticles as adjuvant indicate that changing the route of administration from intramuscular to oral changes the Th1/Th2 balance to increase the impact of Th1. However, primary immunisation, as shown by the subclass analysis, induced a higher Th2 response after oral administration.

Statistically significant changes in the Th1/Th2 balance were not seen after boosting in the experiment where the subcutaneous and the oral route were compared. Indeed, no differences in the subclass ratios were detected after the primary doses or at the end of the immunisation programme. This means that the Th1/Th2 balance of the immune response is similar after oral and subcutaneous administration. However, the subclass ratio in the group immunised subcutaneously was statistically higher than that in the intramuscular group, indicating a higher Th2 influence. This was also supported with a significantly higher serum IgE titre in the group immunised subcutaneously than in the group immunised intramuscularly as IgE has been shown to be co-regulated with IgG1 in mice via the Th2/IL-4 pathway [16].

Thus, it can be concluded that the profile of the immune response obtained with starch microparticles is related to the route of administration. In general, it seems as if oral immunisation gives a profile, which is more similar to subcutaneous immunisation than intramuscular. The mechanisms, which govern the preferential activation of specific Th subsets, appear to be quite complex and are still not completely understood. It has, however, been established that differentiation to Th1 or Th2 phenotypes is largely

controlled by the action of the cytokines IL-12 and IFN- γ to induce a Th1 response, and by IL-4 to direct the response towards Th2 [17,18]. These cytokines are secreted by the T-cells themselves or by the APCs. DCs appear to be the most effective in this respect. Recent studies have found different subsets of DCs in the mouse spleen, Peyer's patches, lymph nodes and skin [19]. These DCs migrate, upon encountering an antigen, to local lymph nodes where they present the antigens to the T-cells. The outcome of the differentiation of the T-cells is dependent not only on the microenvironment (e.g. cytokines) at the actual antigen presenting site [20–22], but could also be dependent on the type of APC presenting the antigen, the amount and nature of the antigen, the presence of adjuvants, the genetic background of the subject (including MHC), and the duration and strength of both T-cell receptor [23] and co-stimulatory signals [24]. It is therefore not surprising that the route of administration affects the differentiation of T-cells.

Particulate antigens and vaccine carriers are preferentially taken up by M-cells in the Peyer's patches [25,26]. Murine Peyer's patches contain a subset of DCs that is more likely to induce Th2-related cytokines than the DCs found in the spleen [27]. The mucosal response induced in the oral groups in the experiments described here was most likely induced by locally produced Th2-related cytokines such as IL-4, IL-5, IL-6 and IL-10. However, DTH testing indicated also a strong Th1 response in all groups tested. This dual Th1/Th2 response may have occurred because of the ability of HSA to give rise to different peptides upon degradation, with distinct abilities to induce either a Th1 or a Th2 response. Several studies have demonstrated that different routes of immunisation can induce different patterns of T and B cell epitope recognition [28,29]. Todryk et al. [30] have demonstrated that the route of administering vaccine (subcutaneous, intraperitoneal, oral) clearly had an effect on both T and B cell epitope recognition within a streptococcal antigen, when the effect of extracellular degradation in the different compartments was taken into consideration. In our study, the route of administration was thought to influence the degradation pattern of the HSA-conjugated starch microparticles. In addition, the subclasses in serum indicated a mixed Th1 and Th2 response with a greater Th2 influence in all groups. A mixed response was also seen in earlier studies after immunisation with the starch microparticles [9,10]. However, the response was more polarised towards Th1 after immunisation with *Salmonella* antigens conjugated to the microparticles than after immunisation with HSA-microparticles in the same strain of mice. This could reflect the influence of the antigen as some antigens can strongly affect the Th1/Th2 balance of the immune response. *Salmonella* antigens are strong immunogens from a pathogen known to be associated with a Th1-type of response [31,32]. HSA on the other hand is considered to be fairly neutral in this respect, i.e. it is unlikely to send specific danger signals to the organism, as seen with some pathogen components which can activate the innate immune response via Toll-like receptors

[33]. HSA was thus considered to be ideal for investigating the relationship between the immune response and the route of administration of the vaccine.

In conclusion, our results show that the profile of an immune response depends on the route of administration. Only vaccination regimens including oral booster doses induced a significant s-IgA response. Oral and subcutaneous immunisations had similar effects on the Th1/Th2 balance. However, this ratio was significantly different for oral and intramuscular immunisations: the Th2 response was stronger after oral primary immunisation than after intramuscular primary administration, while the relative Th1 response was stronger after an oral booster than after an intramuscular booster.

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