Chapter 3

Improvement of the systemic prime/ oral boost stragegy for systemic and local responses

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Chapter 3

Summary

This paper describes oral boost immunisations of primed animals as an alternative oral vaccination strategy. Mice were primed orally (PO), intranasally (IN), subcutaneously (SC), or intraperitoneally (IP) with ovalbumin (OVA) with or without adjuvant. Boost immunisations were given orally with or without cholera toxin (CT) as adjuvant. Prime immunisations induced variable IgA and IgG_1 titres in serum depending on the route. A subsequent oral boost increased these titres. Use of an adjuvant in the priming significantly increased serum IgA and, to a lesser extend, IgG_1. Oral boost immunisation induced significantly higher serum IgA titres in animals primed via the SC, IP and the IN route compared to the PO route. This was independent of the use of CT. Three oral boosts with OVA plus 5 μg CT given in five days to primed mice revealed higher IgA titres compared to single oral boosts and anti-OVA IgA titres in faeces were also detected. Finally, we put together our findings and propose a systemic priming/oral boost strategy in which mice were primed via the SC route with 100 μg OVA plus 50 μg Butyl16-p(AA), and subsequently orally boosted with three doses of 300 μg OVA plus 5 μg CT each.

We concluded that oral immunisation is more effective in IN, SC, or IP primed mice than in PO primed mice, and that the IgA antibody response in serum and faeces can be improved by increasing the immunisation frequency and the use of appropriate adjuvants in primary and boost immunisation. The here-formulated strategy improves the probability of success of oral vaccination. The results are discussed in the light of the development of edible vaccines.

Introduction

Oral vaccination is an attractive but not very efficient way to induce immunity. Despite considerable effort, only few alive oral vaccines are commercially available at this moment. Several studies demonstrated that oral vaccination requires multiple administrations of high doses of antigen, which increases the production costs. Furthermore, oral intake of antigen tends to establish a state of immunotolerance rather than immunity. Obviously, the primary function of the gastro-intestinal tract is not to develop immunological reactions to the various food ingredients entering this organ. In order to obtain significant immune responses, appropriate adjuvants or antigen-delivery systems are used for oral immunisation [1,2]. The co-administration of antigens with bacterial toxins, such as cholera toxin (CT) or heat-labile enterotoxin of *Escherichia coli* (LT) improves the immune response. To deliver non-living antigens to the mucosal immune system, conjugates of antigen and B-subunits of CT or LT, or antigens incorporation into microparticles were used [3-5]. Despite these approaches, oral vaccination with non-living antigen still induces insufficient levels and duration of immunity [1,2,6,7].

Improvement of the systemic prime/oral boost strategy

To improve the efficacy of oral vaccines and to reduce the risk of immunotolerance, we examined the potentials of oral vaccination in mice primed previously by one of the systemic routes. Several authors have shown previously that oral boost immunisation of primed animals is effective in stimulating local and systemic responses. This systemic prime/oral boost strategy was first reported by Pierce and co-workers in 1977 and proved to enhance the enteric immune response to non-replication antigens (CT) and provided long-lasting protection against a subsequent challenge in dogs [8]. The consequences of this immunisation strategy on local and systemic immune responses have been studied further in mice using living antigen [9], OVA [10] and recently using hepatitis B surface antigen [11]. Furthermore, this immunisation strategy has also been applied to birds using replicating antigens [12,13]. In our study, we refined this oral boost concept. Priming conditions such as the type of adjuvant used, route of systemic immunisation, number of immunisations, and dose of antigen were studied. Finally, we present a systemic prime/oral boost strategy with improved probability of successful oral vaccination with non-living antigens.

Materials and methods

Vaccine and adjuvant preparation

Ovalbumin (OVA; A-5503, Grade V, Sigma) was dissolved in saline and mixed with or without one of the following adjuvants:

rCTB: recombinant CTB [10].

Specol: a water-in-mineral oil-emulsion [10,14].

S/W: squalane-in-water emulsion containing 80 g l^{-1} squalane and 20 g l^{-1} Tween 80 [15,16].

SE/S/W: sucrose ester-in-water emulsion containing 8 g l^{-1} sucrose poly fatty acid, 80 g l^{-1} squalane (Merck, Germany), and 20 g l^{-1} Tween 80 (ICI) in phosphate buffered saline (Covaccine BV, The Netherlands).

SL-CD/S/W: sulpholipo-cyclodextrin in a squalane-in-water containing 80 g l^{-1} squalane, 20 g l^{-1} Tween 80, and 8 g l^{-1} SL-CD (Fort Dodge Animal Health, The Netherlands) [16,17].

Butyl16-p(AAA): butyl alkyl-polyacrylate with an esterification grade of 16% (Butyl16-p(AA)) (Fort Dodge Animal Health, The Netherlands) [18].

CT: cholera toxin (CT; C-8052, Sigma).

Animals

Swiss female mice (6 to 8 weeks old) were obtained from Charles River (Sulzfield, Germany) and housed per groups under conventional conditions. All animals were

raised and kept on an OVA free diet. Experimental groups consisted of five to six animals. All animal experiments were held under auspices of the ID-Lelystad BV Animal Experimentation Committee according to the Dutch Law on Animal Experimentation.

Immunisation and sample collection

All oral immunisations (per orally, (PO)) were preceded by overnight fasting of mice (water was provided *ad libitum*) and administered by intragastric intubation of 0.4 ml or 0.5 ml vaccine. Subcutaneous (SC) and intraperitoneal (IP) immunisations were administered in a total volume of 0.1 ml. For intranasal (IN) immunisation, the animals were anaesthetised according to standard methods and 10 μl of vaccine were applied to each nostril.

Experiment 1: Groups of six mice were immunised by the SC or IP route with 100 μg OVA mixed with or without adjuvant. Three or four weeks later, on day 0, mice were given PO boost immunisations of 10,000 μg OVA in 0.5 ml saline. Tail blood serum samples were collected at several time intervals.

Experiment 2: Groups of five mice were immunised by the PO route with 10,000 μg OVA mixed with 5 μg CT in 0.4 ml saline, or by the IN route with 10,000 μg OVA mixed with 5 μg CT in the appropriate volume. Others were immunised by the SC or IP route with 100 μg OVA and 50 μg Butyl16-p(AA) in the appropriate volume. Three weeks later, on Day 0, all mice were given a PO boost with 10,000 μg OVA mixed with 5 μg CT. Boost immunisations were administered according to a 'single dose' schedule (day 21) or a 'triple dose' schedule (day 21, 23, and 25) [19]. Tail blood serum and faeces samples were collected at several time intervals. Fresh faeces pellets were collected and immediately frozen at –20 °C. To prevent degradation by proteases, faeces samples were pre-treated as described earlier [20].

Experiment 3: Groups of five mice were immunised by the SC route with 100 μg OVA and 50 μg Butyl16-p(AA). Three weeks later, on day 0, mice were given a 'triple dose' PO boost with various doses of OVA mixed with 5 μg CT. The doses tested were: 0.01, 0.3, 10, 300, and 10,000 μg. Tail blood serum and faeces samples were collected at several time intervals and processed as described above.

Detection of anti-OVA antibodies

High binding ELISA plates (Greiner, Nürtingen, Germany) were coated overnight at 4°C with 100 μg ml^{-1} OVA (Sigma) dissolved in PBS. Serum and faeces samples were twofold serially diluted in PBS, 0.05% Tween 20, and 1% bovine serum albumin. ELISA was performed as described earlier [20].

Statistical analysis

Antibody titres were expressed as the dilution factor of the sample giving an extinction value of 1 above the background. Geometric mean titres (GMT) of individual 2-log

titres, standard deviation (SD), standard error of the mean (SEM) and antilog (2^{GMT}) values were calculated. Statistical analysis was performed by Student's two-tailed *t*-test. Differences between groups with P value < 0.05 were considered to be significant.

Results

Effects of systemic priming with different adjuvants on the IgG$_1$ antibody response in serum after oral boost

Groups of mice were primed by the IP or SC route and three or four weeks later, on day 0, they were boosted by the PO route. In parallel, groups of naïve mice were immunised solely by the PO route on day 0. PO boost immunisation of primed but
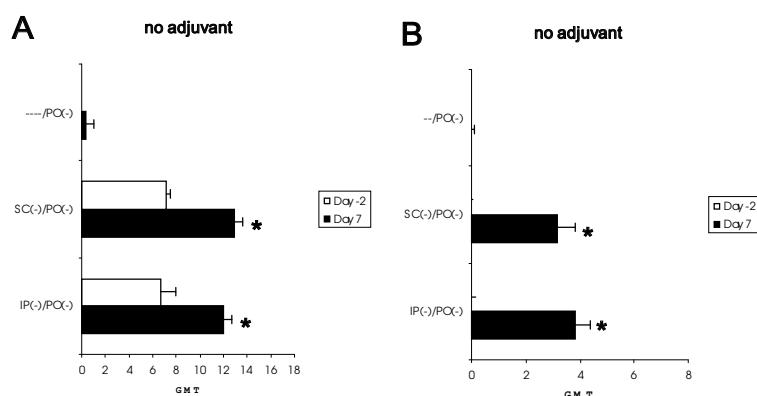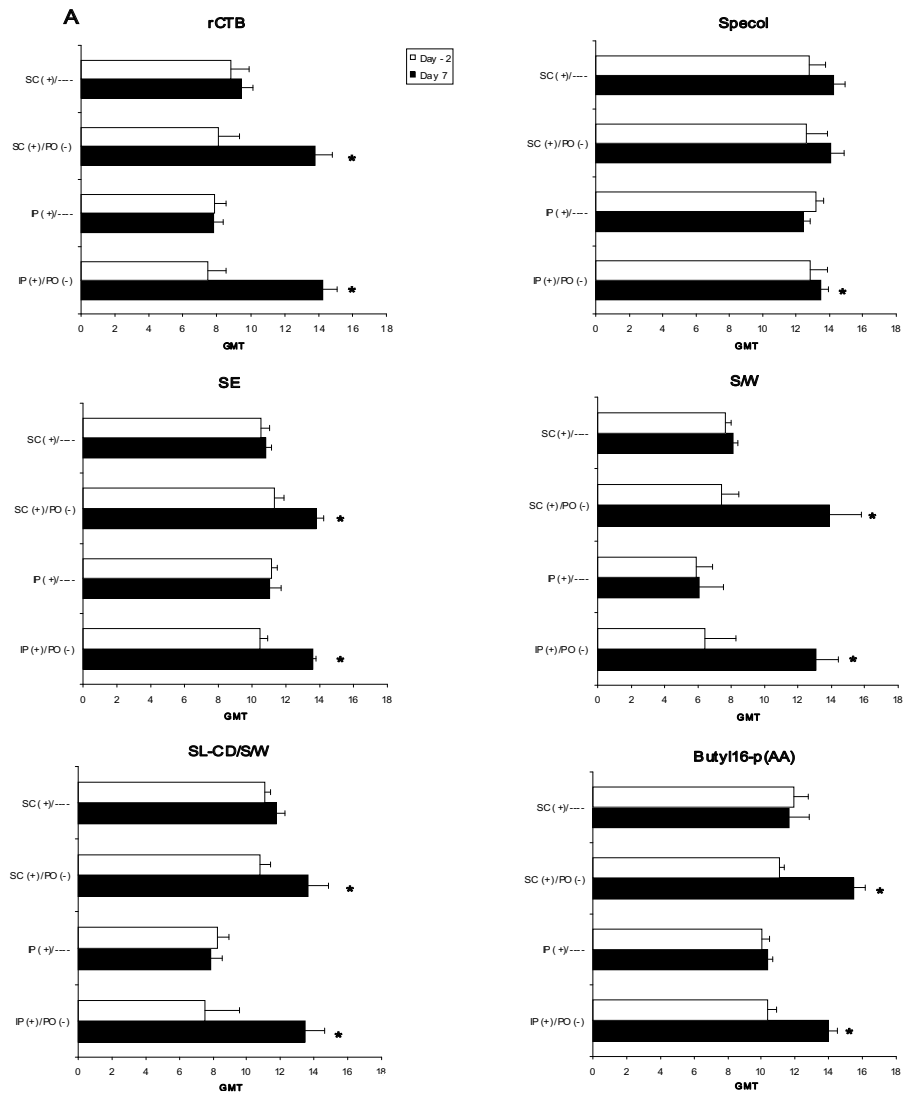

Figure 3.1

Serum IgG$_1$ and IgA after prime and oral boost immunisation without adjuvant. One group of six mice only received an oral boost immunisation (---/PO(-)), other were SC or IP primed. Serum samples were tested undiluted. The data represent IgG$_1$ (A) and IgA (B) in serum after priming (day –2, open bars) and oral boost (day 7, filled bars). The data are shown as GMTs and SD. Statistically significant differences between naïve and primed animals on day 7 are indicated by an asterisk (P < 0.05).

not naïve mice resulted in high antigen-specific IgG$_1$ responses. Without the use of an adjuvant for priming, the PO boost increased the serum GMT 5-fold (Fig. 3.1A).

Systemic priming with adjuvant considerably increased the pre-boost IgG$_1$ titre on day –2 (Fig. 3.2A), except with rCTB and S/W. The titre at 7 days post-boost was considerably increased by PO boosts without adjuvant compared to non-boosted animals, except after SC priming with Specol.

A

Figure 3.2

Serum IgG$_1$ and IgA after priming and oral boost with adjuvants.

The adjuvants rCTB, Specol, Butyl16-p(AA), SE/S/W, S/W, or SL-CD/S/W were used for the parenteral prime immunisation. Groups of six mice were primed only (SC(+)/-- or IP(+)/--), or primed and boosted (SC(+)/PO(-) or IP(+)/ PO(-)). Serum samples were tested undiluted. The data represent IgG$_1$ (A) and IgA (B) in serum after priming (day –2, open bars) and oral boost (day 7, filled bars). The data are represented as GMTs and SD. Statistically significant differences between antibody titres on day –2 and day 7 are indicated by an asterisk (P < 0.05).

Improvement of the systemic prime/oral boost strategy
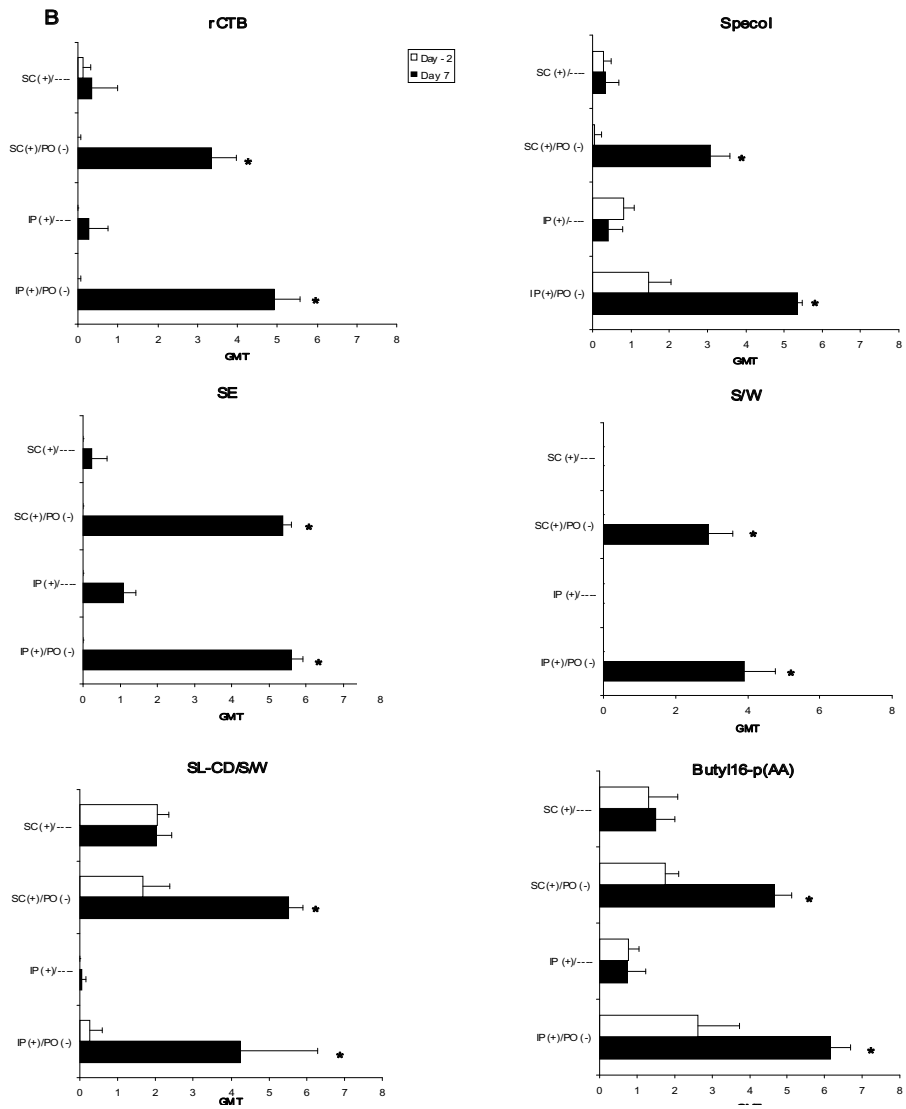
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No significant difference between the SC and IP route was observed, except with SL-CD/S/W, which was more effective after SC administration.

The effect of the PO boost was most pronounced in animals with relatively low pre-boost titres. The serum titres at 7 days post-boost were significantly higher in animals primed via the SC or IP route with rCTB, SE/S/W or Butyl16-p(AA), via the SC route with Specol, and via the IP route with SL-CD/S/W.

Effects of systemic priming with different adjuvants on the IgA antibody response in serum after oral boost

PO boost immunisation of primed but not naïve mice resulted in detectable antigen-specific IgA immune responses in serum (Fig. 3.1B). In a few situations, the addition of an adjuvant to the systemic priming increased the pre-boost IgA titre on day –2 (Fig. 3.2B). Priming via the SC route with SL-CD/S/W or Butyl16-p(AA), or via the IP route with Specol resulted in significantly increased IgA titres in serum.

PO boosts significantly increased serum IgA titres after priming via the SC or IP route with SE/S/W or Butyl16-p(AA), via the IP route with rCTB or Specol, and via the SC route with SL-CD/S/W. No difference between the SC and IP route was observed, except for Specol, which gave significantly higher IgA titres in serum after IP priming.

Effects of priming route on the antibody response in primed mice

In another experiment, we examined which prime/boost immunisation protocol resulted in optimal antibody responses. Mice were primed either systemically by the SC or IP route or mucosally by the IN or PO route on day –21. Butyl16-p(AA) was selected as adjuvant for systemic priming as it induced significantly enhanced serum IgG_1 and IgA titres after PO boost immunisation. To maximise the mucosal immune response, 5 μg CT was used as adjuvant for the IN and PO immunisations. Three weeks later, on day 0, PO boosts were given together with 5 μg CT as adjuvant.

The SC, IP, and IN routes were compared to the PO route in their priming efficiency for an subsequent PO boost immunisation. Pre-boost IgG_1 titres in serum were similar after priming by either route (Fig. 3.3A). Pre-boost IgA titres were significantly higher after priming via the SC or IN route than after the PO route (Fig. 3.3B). Post-boost serum IgG_1 titres on day 7, post-boost serum IgG_1 titres were significantly higher after IN priming than after PO priming. Post-boost serum IgA titres were significantly higher after SC, IP or IN priming than after PO priming. SC priming resulted in significant higher post-boost IgA titres than IP priming. No or very low IgG_1 or IgA responses were detected in faeces (data not shown).

Oral boost of primed mice using a 'triple dose' immunisation schedule

Previously, we demonstrated that three immunisations given in a period of five days was more effective than a single dose[19]. In order to improve the efficacy of the oral

boost immunisation, this so-called 'triple dose' immunisation schedule was applied to the systemic priming/oral boost protocol. According to this schedule, PO boosts were administered on three alternating days, i.e. on day 0, 2, and 4. 5 μg CT was used as adjuvant for the PO immunisations. Compared to a single dose, triple dose PO boosts enhanced significantly post-boost serum IgG_1 titres on day 7 in mice primed by the SC but not by the PO or IP route. IgA titres were enhanced in all groups boosted with triple doses (Table 3.1). In faeces, triple dose PO boosts enhanced significantly the IgA response in all mice, and the IgG_1 response in animals primed by the SC but not by the IP or PO route.

Oral boost with graded doses of OVA

So far, the systemic priming/oral boost protocol consists of SC priming with 100 μg OVA mixed with 50 μg Butyl16-p(AA) followed by a triple dose boost with 10 mg OVA mixed with 5 μg CT. To determine the minimal antigen dose for the oral boost, we have boosted mice with graded doses of OVA. OVA was administered according to the triple dose schedule, at doses of 0.01 μg, 0.3 μg, 10 μg, 300 μg or 10,000 μg per administration mixed with 5 μg CT. Control mice were not immunised. In serum (Fig. 3.4A), significant anti-OVA IgG_1 and IgA titres were induced with 300 μg and 10,000 μg OVA. Antibody responses in faeces were low, and titres were only significant with the two highest doses (Fig. 3.4B).

Discussion

The oral vaccines presently available consist of alive microorganisms. Despite considerable research efforts, the use of non-living antigens has not resulted in effective products. Oral administration of non-living antigens can elicit detectable immune responses, but these are often low. Apparently, exposure of the immune system of the gastro-intestinal tract to non-living antigens is not a very efficient way to trigger responses. Here, we examined the efficacy of oral administration of a non-replicating antigen in systemically (parenterally) primed and naïve animals. The effects of adjuvants used for systemic priming, the route of systemic priming, the immunisation schedule, and the dose of antigen on systemic and local antibody titres have been studied.

Repeated oral immunisation with OVA without adjuvant was not strong enough to induce detectable immune responses in serum. When oral immunisation was preceded by systemic immunisation, significant serum responses were detected. IgA was observed only after the oral boost immunisation. The oral booster increased the IgG1 that was elicited by the priming. We concluded that systemic administration of antigen primes the host for a subsequent contact of antigen with the immune system associated with the gastro-intestinal tract. This is in agreement with earlier reports demonstrating the
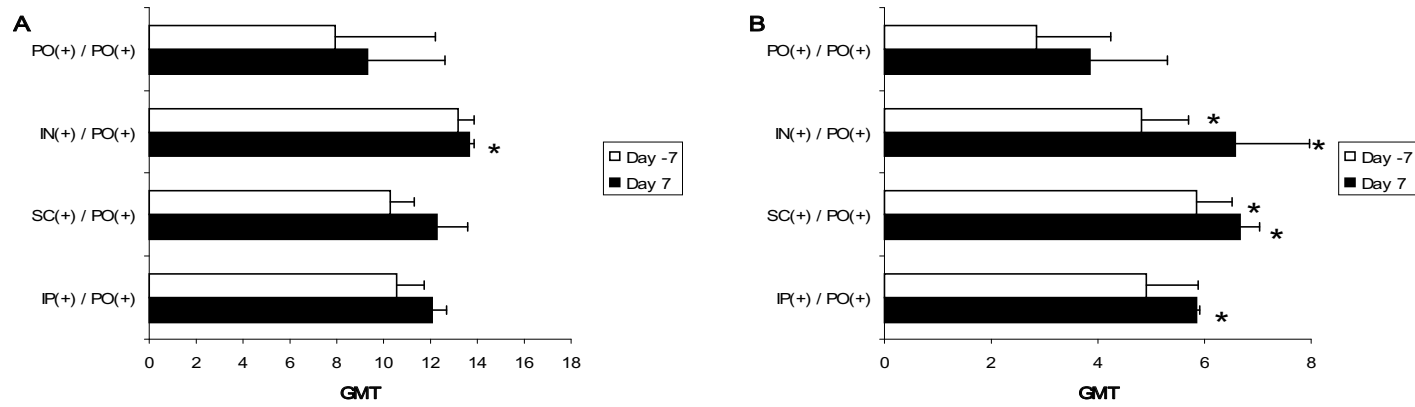
Figure 3.3
Serum IgG$_1$ and IgA after priming via different routes and oral boost immunisation.
Prime immunisations were administered via different parenteral (SC and IP) or mucosal routes (IN and PO) together with an adjuvant. Oral immunisations were given with 5 µg CT. Serum samples were 40 to 400 times diluted for IgG1 measurement and tested undiluted for IgA measurement. All faeces samples were tested undiluted.
The results represented are IgG$_1$ (A) and IgA (B) in serum after priming (day −7, open bars) and oral boost (day 7, filled bars). The data are represented as GMTs ± SD. Statistically significantly higher antibody titres than in PO(+)/PO(+) primed mice are indicated by an asterisk (P < 0.05).

induction of both systemic and mucosal antibody by a combination of systemic and mucosal immunisation [2,8-10,12,13].

Six different adjuvants, which have been proven to be effective for systemic or mucosal responses or both, were selected for systemic priming to evaluate whether the immune response can be further increased. CT and CTB are well-known strong mucosal adjuvants [3]. Specol is a water-in-mineral oil emulsion with a strong activity towards a wide range of antigens. It is similar to Freund's Incomplete Adjuvant, reactogenic and persists at the site of injection [14,21]. S/W [15,16], SL-CD/S/W and SE/S/W are emulsions of squalane-in-water and have considerable adjuvant capacities and low reactogenicity. SE/S/W and SL-CD/S/W are powerful adjuvants with low reactogenicity upon systemic immunisation [16,17]. Butyl16-p(AA) is a water-soluble polymer and has been proved to be effective for both systemic and mucosal immunisation [17,18]. IN immunisation with Butyl16-p(AA) induced high IgA and IgG responses in the lungs and spleen. In general and as expected, the adjuvants enhanced pre-boost serum IgG_1 titres but did not induce detectable IgA responses. Depending on the type of adjuvant and route of priming, oral boosts induced IgA responses in serum and further increased the IgG_1 titre. S/W was the only adjuvant that did not enhance pre-boost IgA titres, but the combination of S/W with the synthetic sulpholipo-derivatives of cyclodextrin (SL-CD/S/W) or with sulpholipo-derivatives of sucrose (SE/S/W) enhanced significantly the immune response after SC administration. SE/S/W and Butyl16-p(AA) were the two adjuvants, which enhanced both IgG_1 and IgA titres in pre- and post-boost serum. Applied in oral vaccines, adjuvants might improve the persistence of the antigen in the gastro-intestinal tract, the targeting of the antigen to the immune system (e.g. by specific binding to epithelial cells or facilitating translocation across epithelial surface), or the molecular context of the antigen or might activate the immune system (e.g. cytokines) [4,22]. However, the exact modes of action of adjuvants are still poorly understood.

The effect of the oral booster was determined on day 7 post-boost. Previous experiments demonstrated that the post-boost antibody titre was maximal 7 days later and did not further increase on day 14 or on day 21 (data not shown). The effect of an oral boost on IgG_1 was evident with all adjuvants, except after IP priming with Specol. The increase in antibody titre was most distinct in animals with relatively low pre-boost IgG_1 titres and not, for example, in animals IP primed with Specol. In the latter cases, the systemic priming revealed high titres and the effect of the oral boost was overshadowed by that of the prime immunisation. Probably, some kind of plateau is reached by strong systemic priming. In addition, mucosal immunisation is not capable of boosting the immune response to high level, but rather to maintain antibody titres at a steady level [23]. Oral immunisation mostly induces T-helper type 2 responses, which was confirmed by our observations that only IgG1 and not IgG2a (data not shown) was induced. Remarkably, IgG2a was even not induced after systemic priming with an adjuvant (data not shown). As compared to IgG_1, the effects of an oral boost on IgA

Table 1: Effects of 'triple dose' oral immunisation

		serum				faeces			
		IgG1		IgA		IgG1		IgA	
priming	booster	Day -7	Day 7	Day -7	Day 7	Day -7	Day 7	Day -7	Day 7
PO(+)	PO(+)	7.9 ± 4.3	9.3 ± 3.3	2.9 ± 1.4	3.8 ± 1.5	< 1	< 1	0.3 ± 2.6	0.0 ± 1.2
SC(+)	PO(+)	10.3 ± 1.0	12.3 ± 1.3	5.9 ± 0.6	6.7 ± 0.3	< 1	< 1	< 1	< 1
IP(+)	PO(+)	10.6 ± 1.2	12.1 ± 0.6	4.9 ± 1.0	5.9 ± 0.1	< 1	< 1	< 1	< 1
3PO(+)	3PO(+)	9.0 ± 1.9	14.1 ± 0.1	3.3 ± 1.1	10.1 ± 1.3[a]	< 1	< 1	< 1	3.4 ± 0.4[a]
SC(+)	3PO(+)	11.5 ± 0.6	14.8 ± 1.9[a]	6.0 ± 0.8	9.9 ± 2.2[a]	< 1	1.8 ± 0.8[a]	< 1	6.0 ± 1.3[a]
IP(+)	3PO(+)	11.0 ± 2.1	15.2 ± 2.3	6.0 ± 1.4	9.0 ± 0.2[a]	< 1	0.1 ± 2.0	< 1	4.9 ± 1.5[a]

The data represent the mean 2-log titre ± SD in serum and faeces 7 days before and 7 days after the oral booster. Significantly higher titres after 'triple dose' immunisation compared to its 'single dose' counterpart
are indicated by an [a].

were more obvious and were found with all adjuvants. This was as expected, since systemic immunisation induces poor IgA responses and the booster immunisations were given via one of the mucosal routes. Because IgA is the most important
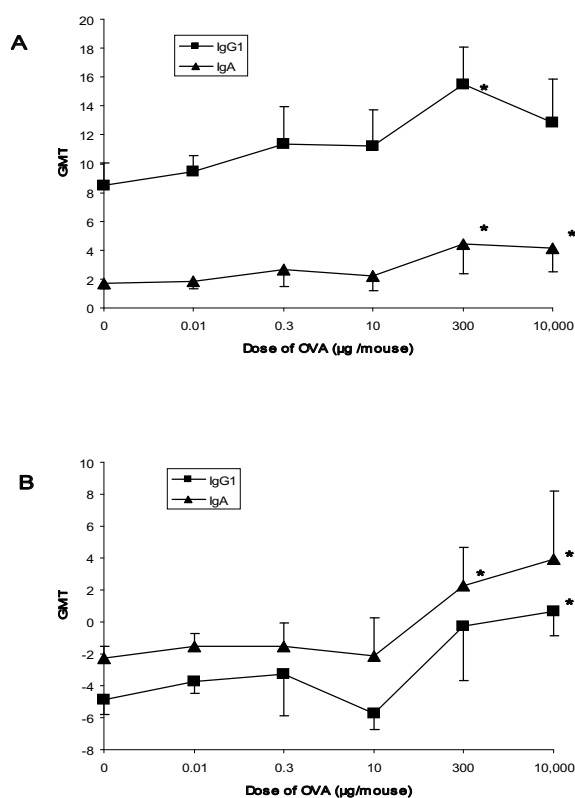
Figure 3.4
The relationship between dose of antigen and IgG_1 and IgA response.
Mice were primed SC with OVA in various doses and Butyl16-p(AA). 'Triple dose' oral boost immunisations were given with 5 μg CT. The data represent the post-boost antibody titres at day 7 in serum (A) and faeces (B) and are depicted against the antigen dose in milligrams per animal. IgG_1 is indicated by squares and IgA by triangles. Statistically significant anti-OVA titres above the background are indicated by an asterisk ($P < 0.05$).

immunoglobulin produced by the mucosal immune system, we focussed our study on this antibody.

Next to the effects of adjuvants, we studied the effects of the route of priming. In order to maximise the immune response, we decided to use Butyl16-p(AA) as adjuvant for the systemic immunisation in the further experiments and CT for the mucosal immunisation. Butyl16-p(AA) was able to stimulate both systemic and mucosal responses and CT is today's best-known adjuvant for oral immunisation. Systemic (SC and IP) and mucosal (IN) priming elicited higher post-boost IgG_1 and IgA antibody responses in serum than PO priming, indicating that solely oral immunisation is less effective than a combination of different routes. All animals were closely observed during immunisation. Nevertheless, swallow of the vaccine after IN immunisation could never be completely excluded. We decided to use the SC route for priming as it is more convenient that IP and better to control.

In contrast to other findings, no antibodies were found in faeces, despite the use of CT as mucosal adjuvant [3]. A single oral boost immunisation with OVA is probably not efficient enough to induce local responses. Manners to further improve mucosal responses in orally boosted animals were considered. Previously, we observed that three oral doses in five days revealed higher responses than single doses [19]. This so-called 'triple dose' immunisation schedule was applied to further increase the effect of the oral boost. As a consequence of this schedule, the antigen dose is three-fold higher but also the exposure of the antigen to the immune system is prolonged. Serum and faeces IgG_1 and IgA titres were significantly higher after 'triple dose' immunisation of naïve and primed mice than after 'single dose' immunisation. Augmentation of IgA by oral boost immunisation was detected in serum and faeces. We concluded that significant local responses can be induced by oral boost immunisation, provided that multiple doses are given, which is in agreement with observations by others [1]. Besides faeces, intestinal scrapings were tested and the number of antibody-secreting cells in the lungs or in the lamina propria of the small intestine were examined (data not shown). No responses could be detected in these samples. Obviously, these samples could only be obtained ate the end of the animal experiments which is may probably be not the optimal time point to measure mucosal immune responses.

The gastro-intestinal tract is originally not designed to react with an immune response towards orally delivered antigens and multiple oral administrations of antigen harbours the risk to establish of a state of immunotolerance [26]. The concept of systemic priming followed by oral boosts prevents the development of oral tolerance. Once the immune system has established an immunological memory to a certain antigen, tolerance is not induced by subsequent feeding of the antigen, not even after increasing the antigen dose and frequency of feeding [27]. Antigen-experienced T cells may be inherently resistant to induction of tolerance, because these cells are less dependent on co-stimulation for their activation than naïve T cells. Primed T cells localise in discrete anatomical

niches where they are capable to sustain their effector functions but are inaccessible for tolerogenic signals [28]. We only examined the humoral responses by determination of the number of antibody-secreting cells in the lamina propria of the small intestine. We did not study the number of T cells in serum or any other compartment of the (mucosal) immune system. These types of studies are of interest in order to elucidate the mechanisms underlying parenteral priming for oral booster immunisation.

The systemic priming/oral boost strategy is already known for several years [8-10,12,13,29] . We attempted to further optimise this concept. The highest systemic and local responses were found after SC priming with 100 μg OVA plus 50 μg Butyl16-p(AA) as adjuvant, and subsequently triple dose boosted with 300 μg OVA plus 5 μg CT. Our refined priming/boost strategy may contribute to the development of more successful oral vaccines.

Chapter 3

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Chapter 3