Chapter 2

A multiple dose immunisation protocol suitable for edible vaccines

Tosca Lauterslager and Luuk Hilgers

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Summary

Frequent administration of oral immunisation has proven to be more successful than single administration. The frequency of feeding edible vaccines, however, is limited by the maximal oral intake, the lack of nutritional value and the possible presence of toxic ingredients. Therefore, we designed a protocol in which the animals received multiple immunisations on three alternating days ('triple dose') and the protocol was compared to single immunisations. Mice were immunised via intragastric (IG) gavage with ovalbumin (OVA) mixed with cholera toxin (CT) and the effects on systemical and local immune responses were determined. Serum IgG1 and IgA titres against OVA after oral boost immunisation given three weeks after primary immunisation were significantly higher after 'triple dose' than after 'single dose' immunisation. Faecal IgA was detected only after 'triple dose' boost immunisation. A second boost did not further increase serum IgG1 and faecal IgA. Antibody responses against CT were also elicited and again, boost immunisations did not further increase this response. We concluded that oral immunisation with multiple doses was more effective than 'single dose' immunisation and it seems practical and efficient for edible vaccines.

Introduction

Several factors affect the immune response upon oral administration of antigen and a few can be manipulated [1]. General complicating factors are degradation of the antigen in the gastro-intestinal tract and the induction of a state of oral tolerance [2,3]. Furthermore, the nature of the antigen strongly determines the outcome of oral immunisation. Oral administration of live pathogens revealed in many cases significant mucosal and systemic immune responses [4]. Oral immunisation with non-living pathogens, subunits or peptides, however, is often inefficient and requires multiple administrations with large amounts of antigen and adjuvant [5]. Another important factor is the immunisation schedule. Chalacombe [6] found that a weekly immunisation did not result in significant responses. Serial immunisations on consecutive days, however, induced sIgA. These and other data suggested that frequency and timing of immunisation are important.

Detailed study on differences between single dose immunisation and multiple dose immunisation on the development of IgA and IgG1 antibodies and their course in time after priming and booster immunisation are not described yet. The goal of the present study was to establish an effective oral immunisation protocol applicable for

edible vaccines for which the frequency of feeding is limited by the maximal possible oral intake.

Materials and methods

Mice

Swiss female mice (6 to 8 weeks old) were obtained from Charles River (Sulzfield, Germany) and housed per groups under conventional conditions. All mice were raised and kept on an OVA free diet. All animal experiments were held under auspices of the ID-Lelystad B.V. Animal Experimentation Committee according to the Dutch Law on Animal Experimentation.

Antigen preparation and immunisation

The antigen preparation tested consisted of 10 mg of ovalbumin (OVA; Grade V, A-5503, Sigma) mixed with 5 μg cholera toxin (CT; C-8052, Sigma) dissolved in 0.4 ml saline. Mice fasted overnight (water was provided *ad libitum*) and were immunised orally

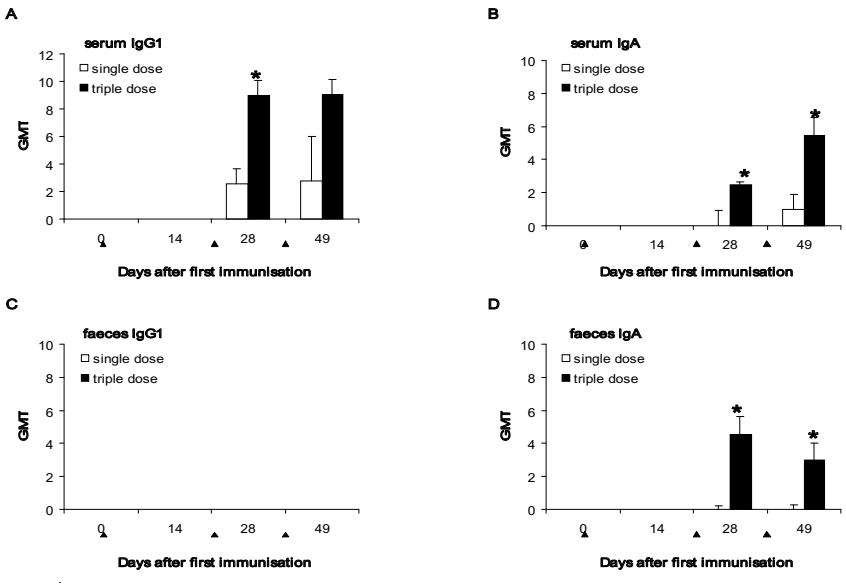

Figure 2.1
Anti-OVA antibody titres after priming and subsequent boost immunisations with 10 mg OVA and 5 μg CT. Arrowheads mark days of immunisation. An asterisk marks statistically significant differences between 'single' and 'triple dose' oral immunisations. The data represent GMTs and SEMs of serum IgG1 (A), serum IgA (B), faeces IgG1 (C) and faeces IgA (D).

by intragastric intubation with OVA plus CT on day 0, 21, and 42 ('single dose') or on day 0, 2, 4, 21, 23, 25, 42, 44, and 46.

Collection of faeces and serum samples

Pre-immune tail blood serum and faeces samples were collected before the first immunisation and on day 14, 35, and 49. Fresh faeces pellets were immediately frozen at –20 °C. Before testing, faeces pellets were treated as described elsewhere to prevent degradation [7].

Detection of anti-OVA and anti-CT antibodies

High binding ELISA plates (Greiner, Nürtingen, Germany) were coated overnight at 4°C with 100 μg ml^{-1} OVA or 2 μg ml^{-1} CT dissolved in PBS. ELISA was performed as described earlier [7]. 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 and standard error of the mean (SEM) 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. Extinctions below detection limit were considered to have a GMT of -10.

Results

Antibody responses against OVA

Neither 'single dose' nor 'triple dose' oral priming with OVA plus CT resulted in detectable anti-OVA immune responses in serum or faeces (Fig. 2.1). 'Single dose' oral boost immunisations induced anti-OVA IgG1 and IgA titres in serum, and serum IgG1 was significantly higher compared to pre-immune serum on day 28. 'Triple dose' oral boost immunisations induced significantly higher antibody titres in serum (IgG1 and IgA) and in faeces (IgA) compared to pre-immune titres. Second boost immunisations administered on day 42, or at day 42, 44, and 46, further increased serum IgG1 but not serum IgA or faecal IgA, but only after 'triple dose' boost immunisations. Antibody titres were significantly higher (IgG1 on day 28 and IgA on day 49) and in faeces (IgA on day 28) after 'triple dose' immunisation.

The number of responder mice and non-responder mice on day 49 using each immunisation protocol is represented in Table 2.1. Mice were considered to be responding when the GMT titre was at least 1. In serum, 4 out of 4 and 5 out of 5 'triple dose' immunised mice had increased IgG1 and IgA responses, respectively, while 3 out of 5 mice responded with faecal IgA. After 'single dose' immunisation, 4 out of

5 mice had positive serum IgG1 and 3 out of 5 had positive serum IgA responses. None responded with faecal IgA. Also, the GMTs on day 49 of all mice and of only the responding mice are represented in this table. Significantly higher GMTs after 'triple dose' immunisation compared to 'single dose' immunisation are indicated with an [a]. Non-responding 'single dose' immunised mice were not responsible for the differences between the two immunisation protocols.

Antibody responses against CT

Both 'single dose' and 'triple dose' oral priming with OVA plus CT resulted in anti-CT IgG1 in serum (Fig. 2.2), but serum IgA was observed only after 'triple dose' priming. Antibody titres were significantly higher after 'triple dose' prime immunisation. Boost immunisations did not further increase serum IgG1 or IgA and no differences between the immunisation protocols were found after the boost immunisation. Antibody responses in faeces were not determined.

Discussion

After oral immunisation with OVA plus CT, OVA-specific IgG1 and IgA could be measured in serum, and 'triple dose' immunisation revealed significantly higher antibody titres than 'single dose' immunisation, but differences were not always significant (Fig. 2.1). CT was necessary as adjuvant as OVA without CT did not evoke detectable

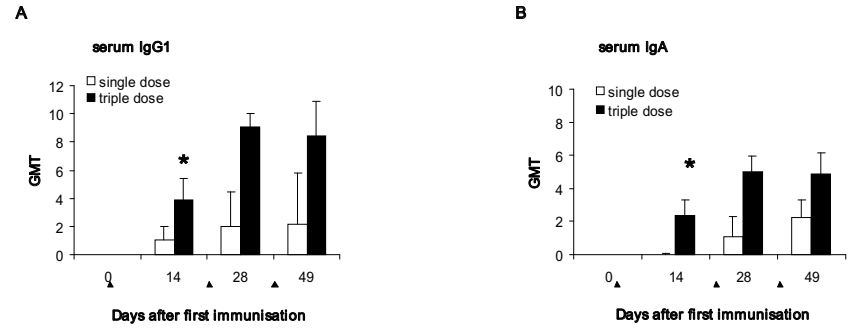

Figure 2.2
Anti-CT antibody titres after priming and subsequent boost immunisations with 10 mg OVA and 5 μg CT. Arrowheads mark days of immunisation. An asterisk marks statistically significant differences between 'single' and 'triple dose' oral immunisations. The data represent GMTs and SEMs of serum IgG1 (A) and serum IgA (B).

Table 2. 1 Responding and non-responding animals on day 49

			all tested mice			responding mice		
	immunisation protocol	# of responding mice / # of tested mice	n	GMT	SEM	n	GMT	SEM
serum IgG1	single dose	4/5	5	2.8	3.2	4	6.0	0.6
	triple dose	4/4	4	9.0	1.1	4	9.0	1.1
serum IgA	single dose	3/5	5	1.0	0.9	3	2.2	0.5
	triple dose	5/5	5	5.5	1.1	5	5.5[a]	1.1
faeces IgA	single dose	0/5	5	-1.0	1.3	0		
	triple dose	3/5	5	3.0[a]	1.1	3	4.6[a]	0.8

The number of responding mice of the total number of tested animals per group is given. Mice were considered to be responding when the antibody titers was > 1. The GMTs and SEMs of the tested mice (responding and non-responding mice) and of the responding mice are represented. Significantly differences between 'triple dose' and 'single dose' immunisations are indicated with an [a].

responses (data not shown). Both 'triple dose' and 'single dose' immunisation induced antibodies to CT, but differences between the protocols were only observed after prime immunisation.

'Triple dose' immunisation was able to induce systemic as well as local antigen-specific antibody responses, whereas 'single dose' immunisation only raised detectable antibodies in serum. In faeces, OVA-specific IgA but not IgG1 was induced. IgA was the predominantly produced immunoglobulin by mucosal tissues. Thus, we expected to find IgA and not IgG1 in faeces. Our results were confirmed by ELISPOT, in which we observed more IgA than IgG1 antibody secreting cells in the lamina propria [unpublished results]. But strong mucosal immunogens like CT and CTB are indeed able to elicit local IgG1 responses [8].

Primary 'triple dose' immunisation was sufficient to induce anti-CT but not anti-OVA antibodies in serum and faeces. Boost immunisations were necessary to induce detectable antibody titres. Second boost immunisations further increased anti-OVA serum IgA titres, but did not further increase serum IgG1 or faeces IgA titres, suggesting that these latter reached a plateau level. Anti-CT titres already reached a plateau after priming. The observation of a plateau suggested that a secondary reaction of the immune system towards boost immunisation with an antigen does not occur. This raised the question if memory is indeed induced in the mucosal immune system. Cebra reported that cells in the germinal centres of Peyers Patches (PP) are transient and that successful secretory IgA responses attenuated the stimulation by secondary mucosal challenge [9]. This might explain why traditional boost responses were not induced after oral immunisation. Our findings indicated that memory cells were formed after 'triple dose' priming, but that the extent of memory triggering was different for each antibody isotype, each compartment of the immune system, and the antigen used. CT is more immunogenic upon oral immunisation compared to OVA and this might explain why differences between 'single -' and triple dose' disappeared after boost immunisations.

Oral boost immunisations were given three and six weeks post-priming. Seven days after the last booster, all 'triple dose' immunised mice responded with anti-OVA IgG1 and IgA titres in serum, while after 'single dose' immunisation, few animals remain non-responding. In faeces, 3 out of 5 of all 'triple dose' immunised mice responded, while in the 'single dose' immunised group no mice responded. The non-responding animals were not responsible for the significant differences between 'single dose' and 'triple dose' immunisation (Table 2.1). Thus, the 'triple dose' immunisation protocol did not only increase the mean antibody titre, but also increased the number of responding animals.

The 'triple dose' oral immunisation protocol as presented in this paper, resulted in higher antigen-specific antibody titres against non-live antigens than to 'single dose' immunisation, most probably due to the extended exposure of the antigen to the mucosal immune system. A major problem in oral immunisation is the degradation of antigen by the gastrointestinal tract, and prolonged exposure of antigen might give the mucosal immune system more time to respond. Frequent oral administration can enhance the efficacy of edible vaccines. The frequency of feeding edible vaccines, however, is limited by the maximal oral intake, the lack of nutritional value and the possible presence of toxic ingredients. The here proposed 'triple dose' protocol was developed for the use with transgenic potatoes as edible vaccines [7]. Since potato-produced vaccines contain less nutrition than standard food and a fasting period is involved, immunisation on alternating days provides mice 24 hours to recover from immunisation at the disposition of standard food.

In the study presented here, the antigen dose of each immunisation was equal, which meant that the 'triple dose' treated mice received a three times higher priming dose than 'single dose' immunised mice. Future studies must determine whether the antigen dose can be divided over the three immunisations days to diminish the risk of antigen-overdose, like toxicity.

'Triple dose' oral immunisation has been proved to be effective in inducing systemical and mucosal immune responses and can be applied in feasibility studies with edible vaccines and to gain more insights in the various aspects of oral immunisation.

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