Chapter 6

Efficacy of oral administration and oral intake of edible vaccines

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Chapter 6

Summary

To evaluate whether vaccine administration via intragastric gavage is indicative for the outcome of edible vaccines, mice were orally immunised with ovalbumin (OVA) mixed with or without *Vibrio cholerae* toxin (CT) in various compositions via various routes: 1) OVA dissolved in saline and administered intragastrically ('ig'); 2) OVA mixed with food extract and administered ig ('food ig); 3) food chow absorbed with OVA dissolved in saline and fed to the animals ('food'); and 4) OVA dissolved in saline and administered via drinking bottles ('drinking'). When given to naive mice, 'ig' and 'food ig' but not 'food' or 'drinking' induced anti-OVA IgG1 responses in serum, but oral boost immunisations were necessary. Serum IgA was not induced. Oral boosting of subcutaneously primed mice enhanced the IgG1 and IgA response in serum regardless of the route of immunisation or the vaccine composition. CT did not dramatically enhance the immune response. All immunisation routes except 'drinking' induced antigen-specific IgA antibody secreting cells in the lamina propria of naive mice. But antigen-specific antibody responses in faeces were not observed.

We concluded that oral (ig) administration is distinct from oral intake. The composition of the vaccine (food or saline) did not influence oral administration. We thus suggested that the route of administration greatly influenced the outcome of oral immunisation. Although oral administration is a well-accepted route to test the potentials of oral vaccines, our study demonstrated that it is merely indicative for the effectiveness of edible vaccines. Studies on the feasibility of edible vaccines should thus be performed by eating the vaccine.

Introduction

Oral vaccination is regarded to be a safe and simple alternative for parenteral administered vaccines. When produced in edible plants or parts thereof, the production costs of such a vaccine can be reduced considerably, increasing the availability and use for both human and animals [1]. Furthermore, production of vaccines in plants eliminates the risk of contamination with animal pathogens such as viruses and prion proteins [2]. The first reports on oral vaccination were published when molecular biological techniques were scarcely available. These studies were performed via intragastric (IG) gavage or intraduonenal (ID) immunisation [3,4]. At that time, many researchers already speculated on the development of edible vaccines and the results of IG or ID immunisation were thought to be predictable for the efficacy of edible vaccines. At present, only few oral vaccines are licensed and available and all are based on live microorganisms [5].

The increasing knowledge on the virulence factors of pathogens allows the development of non-living vaccines that are regarded to be safer than live vaccines [2]. In general, non-living vaccines are poor immunogens and adjuvants are required. The

situation becomes even worse when such vaccines are delivered orally since passage through the gastrointestinal (GI) tract involves many degradation steps. Only a fraction of the initially administered material will finally arrive in the gut. It must then still pass the epithelium in order to elicit an immune response. Degradation can be partially overcome by encapsulation of the antigens. In transgenic plants, plant cells can protect the antigen against the acidic environment of the stomach [5,6] and proteolysis in the GI tract.

Recently, we have reported studies on oral immunisation of mice with potato tubers expressing the B-subunit of *Escherichia coli* heat labile toxin (LTB). We observed that feeding intact tuber was less effective than IG gavage of tuber extract containing a similar antigen dose. We then suggested that the route between mouth and stomach or the vaccine formulation is a crucial factor for the outcome of oral immunisation [7]. In the present study, we evaluate both possibilities. Mice were orally immunised with plain ovalbumin (OVA) or OVA incorporated in standard mice chow in order to mimic edible vaccines.

Materials and methods

Mice

Swiss female mice (6 to 8 weeks old) were obtained from Charles River (Sulzfield, Germany). Animals immunised via intragastric (ig) gavage were housed per group under conventional conditions. Animals immunised by food or drinking water were housed individually. 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

Four antigen preparations were tested:

1) Ovalbumin (OVA; Grade V, A-5503, Sigma) was dissolved in saline at a final concentration of 10 mg per 0.4 ml with or without 5 μg cholera toxin (CT; C-8052, Sigma) and administered via ig gavage ('ig).
2) A food extract of standard food chow dissolved in saline was made. Subsequently, OVA was dissolved in this food extract at a final concentration of 10 mg per 0.4 ml with or without 5 μg CT and administered via ig gavage ('food ig').
3) OVA was dissolved in saline at a final concentration of 10 mg per 0.1 ml with or without 5 μg CT and added to standard food chow of about 1 g until completely absorbed. A single treated chow was given to individual mice (food).
4) OVA was dissolved in saline at a final concentration of 50 mg OVA per 50 ml with or without 25 μg CT. Standard drinking bottles were filled with 50 ml of this

antigen preparation and given to individual mice ('drinking'). The drinking bottles were weighed before and after immunisation.

Mice were fasted overnight before each oral immunisation (water was provided *ad libitum*). The groups immunised via the ig route, received standard food 2 h after gavage. The 'food' and 'drinking' preparations were given for 24 h after which the animals received standard food.

Groups of naive mice were primed orally on day 0, 2, and 4, and boosted orally on day 21, 23, and 25. Other groups were primed subcutaneously (sc) on day 0 with 100 μg OVA and 50 μg Butyl16-p(AA) in 0.1 ml phosphate buffered saline and subsequently boosted orally on day 21, 23, and 25.

Collection of faeces and serum samples

Serum and faeces samples were collected before immunisation and on day 14 and 35. Four to six fresh faeces pellets per mouse were collected and pre-treated as described previously [7].

Detection of antibody secreting cells by ELISPOT

From some groups, lamina propria lymphocytes were isolated from the small intestine and OVA-specific antibody producing cells (APC) were determined by ELISPOT as described before [8].

Detection of anti-OVA antibodies by ELISA

High binding ELISA plates (Greiner, Nürtingen, Germany) were coated overnight at 4°C with 100 μg ml^{-1} OVA dissolved in PBS (pH = 7.4). ELISA was further performed as described earlier [7]. Antibody titres were expressed as the dilution factor of the sample giving an extinction value of 1.0 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

Oral immunisation of naive mice

All 'food' immunised mice ate the treated chow within 24 hours. The 'drinking' groups drank 10 ml on average, which corresponded with an oral intake of 10 mg OVA. Oral priming of naive mice did not evoke OVA-specific IgG1 or IgA antibodies in serum. Oral boosting induced OVA-specific IgG1 antibodies on day 35 after 'ig' or 'food ig' administration but not after oral intake of food or drinking water (Fig. 6.1A). The differences between 'ig' and 'food ig' were not significant. Serum IgA antibodies were

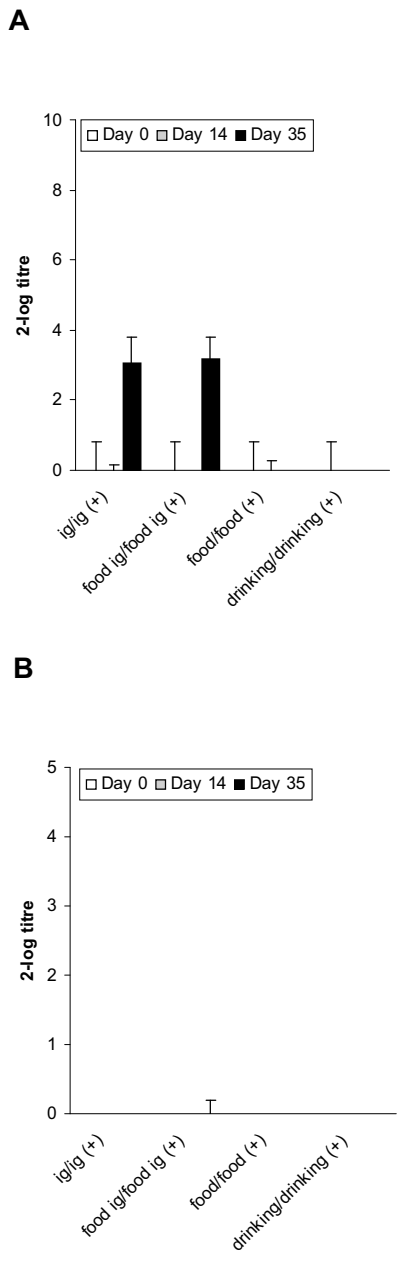

Figure 6.1
The immune response after oral immunisation of naive mice. Naive mice were primed on day 0 and boosted on day 21. CT was used as adjuvant. Samples were collected prior to immunisation. Anti-OVA specific IgG1 (A) and anti-OVA specific IgA (B) in serum. The mean 2-log titre ± SEM for five mice in each group is shown.

not detected after the boost (Fig. 6.1B). No antibody responses were detectable in faeces (data not shown).

Oral immunisation of primed mice

Subcutaneous (sc) priming of mice with OVA plus Butyl16-p(AA) as adjuvant elicited anti-OVA IgG1 but not IgA in serum (Fig. 6.2A and 6.2B). Subsequent oral boosting significantly increased the IgG1 titre compared to non-boosted animals regardless of the route of immunisation or the vaccine composition. The IgA titre was increased in all groups except in the sc/'food ig'(-) and sc/'food'(+) groups, but the titre was only significant in 'ig' boosted mice. Addition of CT only significantly increased the immune response after 'drinking' immunisation.

ELISPOT analysis of lymphocytes of the lamina propria of the small intestine

The presence of OVA-specific antibody secreting cells (ASCs) in the lamina propria of the small intestine were measured only in orally primed and boosted animals. Anti-OVA IgA but not IgG1 ASCs were observed (Fig. 6.3). The highest number of anti-OVA ASCs were observed after 'food ig' immunisation, but 'ig' and 'food' immunisation also induced significant antigen-specific IgA ASCs.

Discussion

Previously, we demonstrated that antigen-specific immune responses after feeding of LTB-expressing potato tubers were weaker than after intragastric (ig) gavage of tuber extract, although the amount of LTB in intact tubers was 30 times higher [7]. We suggested that the method of administration caused these differences. Protein degradation in the gastrointestinal (GI) tract is an important factor in the outcome of oral vaccination. At the beginning of the GI tract, mastication breaks food into smaller pieces, thereby aided by proteolytic enzymes in the saliva. Subsequently, the food suspension is transported to the stomach with its low pH and finally arrives at the small intestines, where the M-cells reside. In the experiments described here, mice were immunised orally with different formulations of ovalbumin (OVA) via different routes. By addition of a fasting period, we assumed that the length of stay in the stomach was similar in all animals. And antigen administered via food or drinking water but not via ig gavage was subjected to the passage from mouth via oesophagus into the stomach.

In a first experiment ig gavage with OVA dissolved in saline ('ig') was compared to immunisation of mice with chow added with the antigen ('food'). In accordance with our observations with transgenic potatoes [7], we observed that 'ig' immunisation was more effective than 'food' and, in contrast to the transgenic potato study, the antigen doses were similar (data not shown). However, besides the difference in route, the vaccine composition was an additional difference.

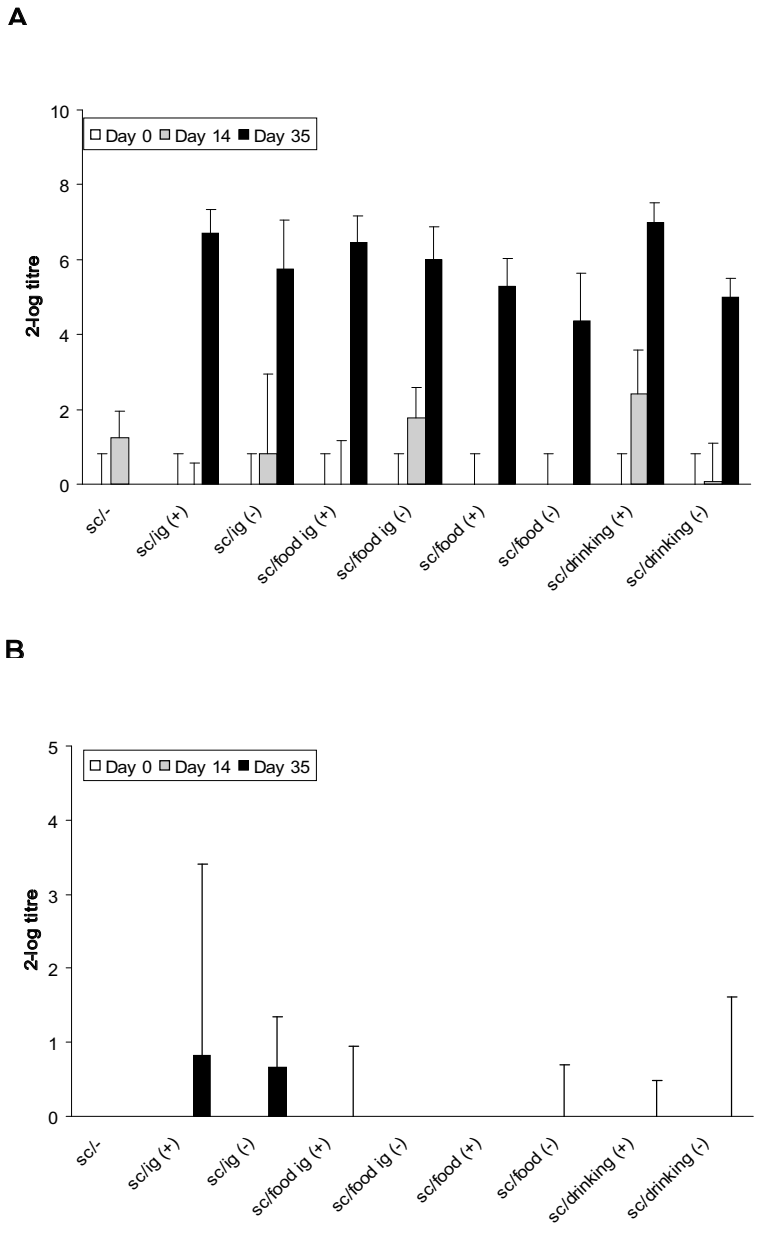

Figure 6.2
The immune response after oral immunisation of primed mice. Mice were primed subcutaneously on day 0 and orally boosted on day 21. Control groups were not boosted (sc/-). CT was used as adjuvant in groups indicated with an +. Samples were collected prior to immunisation. Anti-OVA specific IgG1 (A) and anti-OVA specific IgA (B) in serum. The mean 2-log titre ± SEM for five mice in each group is shown.

To determine the role of food components in oral immunisation, we repeated the experiment with two additional groups: one group received the antigen via drinking water ('drinking') and another via ig gavage of the antigen dissolved in an extract of mice feed ('food ig'). Again, ig gavage was more effective than 'food' immunisation regardless of the vaccine composition (saline or chow extract). ig gavage was also more effective than 'drinking' immunisation. This corresponded with findings of Felder *et al* who directly injected microparticles into the mouth of pigs instead of IG gavage and observed no immune responses [9].

Surprisingly, in subcutaneously (SC) primed mice, 'drinking' was more effective than 'food' immunisation. This suggested that chow components still might have played a role in the outcome of oral vaccination. The primary task of the GI tract is absorption of nutrition out of food and under normal conditions, the body is tolerant against dietary antigens and immunological reactions are preferably prevented [10]. Perturbation of the balance in the gut may lead to unwanted diseases like food allergy, Crohn's disease, and coeliac disease as a result of breakdown in oral tolerance [11]. Taking the natural function of the GI tract into account, it is not remarkable that OVA mixed with food is less effective in evoking an immune response than OVA dissolved in an aqueous phase. The body probably recognises OVA mixed with food as 'normal' food, and does not react on it. On the other hand, 'food IG' immunisation evoked serum IgG1 and high numbers of IgA ASC. This suggested that orally administered antigens were only recognised as food when it follows all processing steps, including passage through the mouth and oesophagus. The route of administration is probably a crucial factor in the outcome of oral vaccination. 'Food' induced significant numbers of APC, so it is not likely that oral tolerance was induced. Future studies must assess whether 'food' immunisation is not immunogenic or induce oral tolerance instead. Our results corresponded with observations of Klipper *et al.* who reported that administration of bovine serum albumin (BSA) in solution induced immune responses whereas feeding of BSA powder mixed with standard food induced tolerance [12,13]. In contrast to us, Klipper *et al.* concluded that the physical form of an antigen was an important factor for oral immunisation.

We fasted the animals to reduce duration of stay in the stomach. After fasting, food is released by the stomach as a bolus. This might have influenced the 'food IG' and 'food' immunisations, but not the other two. The efficiency of vaccine take up out of a bolus may be more difficult. This may be another explanation of the reduced effectiveness of 'food' immunisation, and must be also taken into account when designing an edible vaccine.

Similar to previous experiments of our group [7], oral immunisation of naive mice was less effective than oral boosting of subcutaneously (SC) primed mice. Although all naive mice were immunised with cholera toxin (CT) as adjuvant. Subcutaneous priming with Butyl16-p(AA) as adjuvant triggered the immune system for an oral booster. When

the immune system was sufficiently triggered, all administration routes boosted the immune response, regardless of the vaccine composition and the use of CT.

Surprisingly, IgA was not the predominant immunoglobulin induced by oral immunisation and IgG1 was also induced. In serum, very low IgA titres were measurable, but that was consistent with the observations that IgA is predominantly present in mucosal secretions. This was confirmed by ELISPOT, which revealed that IgA and not IgG1 antibody secreting cells (ASCs) were present in the lamina propria of the small intestine.

Oral immunisation by oral intake of food an attractive concept because of its ease of administration. Various studies have been reported the use of virus-like-particles (VLPs) or non-living vaccines expressed in plants [7,14,15]. Preliminary human trails have been published [16,17]. However, the development of transgenic plants is laborious and time-consuming. Anticipating to future edible vaccines, studies are performed by oral administration of antigen by IG gavage. The present study demonstrated that oral administration is distinct from oral intake of food and may overestimate the efficacy thereof.

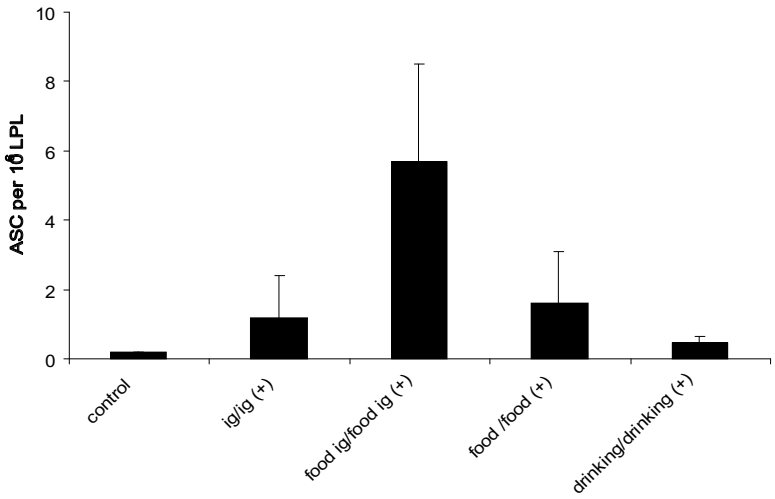

Figure 6.3
Detection of OVA-specific antibody secreting cells (ASC) in the lamina propria lymphocytes of orally immunised naive mice. Control mice were not immunised. The mean number of ASC per 10^6 lamina propria lymphocytes (LPL) $\pm$ SEM are shown.

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