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**Dose and timing requirements for immunogenicity of viral poultry vaccine antigen:  
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Figure 1.

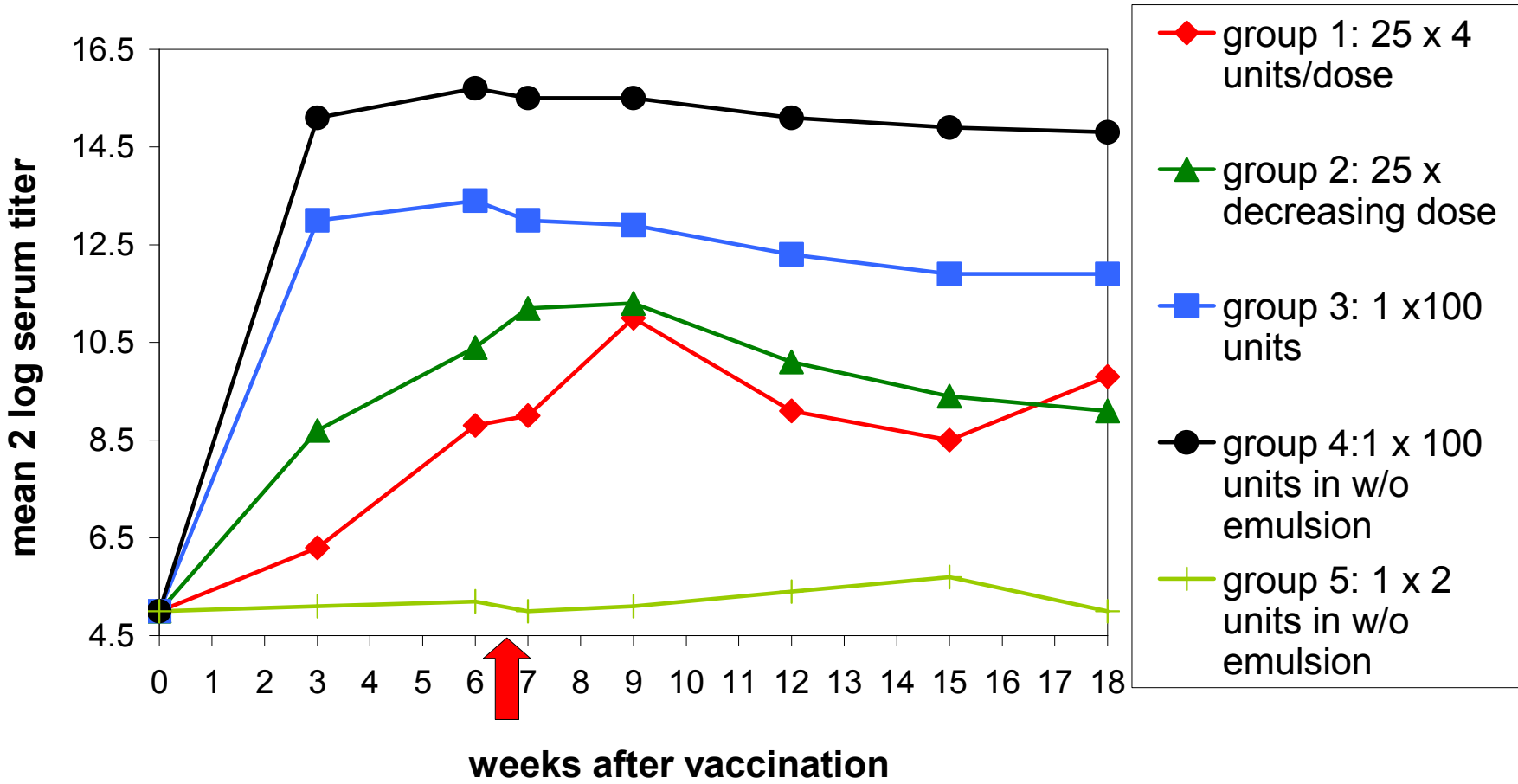
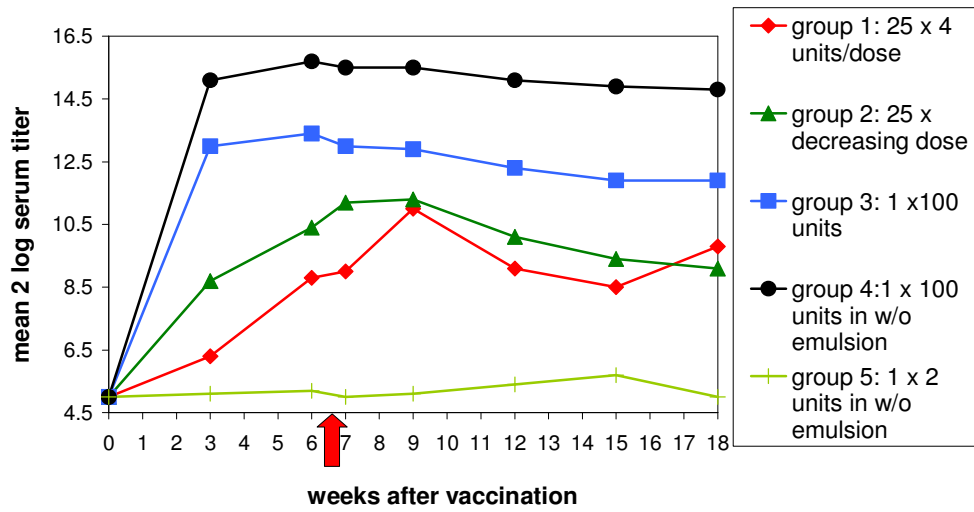


Figure 1.



Jansen et al., Figure 1

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Dose and timing requirements for immunogenicity of viral poultry vaccine antigen: investigations of emulsion-based depot function

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## Dose and timing requirements for immunogenicity of viral poultry vaccine antigen: investigations of emulsion-based depot function

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### Summary

The release requirements for vaccine antigens (Ags) delivered by adjuvants with presumed depot function are poorly understood. Water-in-oil (W/O) emulsions are routinely used in many poultry vaccines. They strongly activate antibody production, and are regarded as a depot from which Ags are slowly released, resulting in prolonged antigen residence. However, from earlier studies we concluded that W/O adjuvant activity is partly based on the immunostimulatory activity of the oil phase. Here we assessed the dose and regimen requirements for viral antigen in immunization experiments in chickens. Three-four week-old White Leghorn chickens were repeatedly injected with inactivated infectious bursal disease virus (IBDV) antigen over 48 days. Our aim was to compare the antibody responses in repeatedly injected animals, receiving fractioned doses of antigen, to the responses in animals receiving only one injection of the full dose of Ag formulated in either a W/O emulsion or in saline. We observed that repeated administration of small amounts of Ag results in a gradual increase of specific humoral immune responses during the immunization regimen. Immunization with a higher first dose evoked an early higher antibody response, which however, reached a similar plateau level at the end the regimen. When compared to lower first dose regimens a slow decline of serum antibody titre following two weeks after the ending of Ag injections indicated that

repeated injection of small doses of Ag indeed mimic the efficacy of depot-forming adjuvants. All regimens of fractioned antigen in saline however, proved less effective, when compared to a single dose vaccination of the cumulative amount of antigen formulated in a W/O emulsion. From our data we confirm that W/O emulsions are very effective vaccine vehicles for improving antigen-specific humoral responses in chickens, due to a combination of antigen residence-prolonging activity and direct immune stimulation.

## Introduction

Incorporation of vaccine antigens (Ags) into the aqueous phase of a water-in-oil (W/O) emulsion as a means of immunopotentiality was initially discovered in 1916, when Le Moignic and Pinoy vaccinated mice with inactivated *Salmonella typhimurium* in an emulsion of water and Vaseline oil (Le Moignic, 1916). Broad introduction of W/O emulsions followed in 1937 by Freund. W/O emulsions are known to strongly activate antibody production. This is partly based on the immunostimulatory activity of the oil phase. A range of lipid substances such as hexadecane, squalene, methyl oleate or pristane, possess adjuvant activity for a variety of antigens (Freund, 1951; Whitehouse *et al.*, 1974). Especially non-metabolizable mineral oil, the lipid substance of Freund's W/O emulsion, shows strong immunopotentiating activity. When comparing identical emulsion compositions, varying only in the type of oil, non-metabolizable mineral oil generally evokes higher antibody responses than biodegradable oil, such as a Medium Chained Triglyceride (Jansen *et al.*, 2005; 2006).

Immune activation by W/O emulsions is likely to be based on the triggering of an inflammatory response at the injection site that leads to attraction and activation of antigen presenting cells (APCs) by provision of critical immune responses activating signals (Dupuis *et al.*, 1998; Schijns, 2000), which are not defined at the molecular level. Recently it has been shown that Toll-like receptors are not the essential receptors to trigger antibody responses where classical adjuvants such as Freund's or alum are concerned (Gavin *et al.*, 2006). Apart from these direct immunostimulatory effects W/O emulsions are regarded as antigen (Ag) delivery systems. They are



assumed to act as an inert depot from which the emulsified Ag is slowly released for a prolonged period of time to supply the site of antibody production, a process which is considered to be a crucial factor in maintaining immune responses. Indeed, W/O emulsions show depot characteristics for incorporated Ags, because in this two-phase emulsion system the Ag-containing aqueous phase is dispersed as small droplets in the continuous oil phase. By contrast, oil-in-water (O/W) emulsions, with the Ag-containing water phase as the continuous phase, show a different release profile of formulated antigen. After minimal or no delay it is quickly released (Aucouturier *et al.*, 2001; Jansen *et al.*, 2006).

Herbert (1968) published an elegant study on the mode of action of W/O emulsion adjuvant for antibody production in mice. Using highly purified ovalbumin (OVA) in mineral oil emulsions, he concluded firstly that high antibody responses were observed in mice inoculated with even the smallest quantity of OVA in W/O emulsion; dilution of W/O emulsions with more oil seemed to increase the initial antibody formation. Secondly, no adjuvant effect was observed in the case of the sole presence of emulsifiers that are used in the formulation of W/O emulsions. In addition, residual W/O emulsion depots, found encapsulated at the injection site in mice 271 days after inoculation, when collected and administered subcutaneously to naive mice, contained sufficient Ag to stimulate an antibody response in these newly inoculated mice. This suggested that not all Ags are released from the injection site during the 271 day period. Herbert attempted to mimic a slow release mechanism by daily injections of tiny doses of OVA alone in mice (Herbert, 1966) and noted an antibody production profile similar to a single full dose OVA formulation in W/O emulsion. Interestingly, the antibody level in the circulation dropped soon after the daily injections were stopped.

The present study revisited the dose and regimen requirements for viral Ag in chickens, a species routinely immunized with W/O emulsion vaccines. Three-four week-old chickens were repeatedly injected with inactivated infectious bursal disease virus (IBDV) antigen for 48 days. Our aim was to compare the antibody responses in repeatedly injected poultry, receiving fractionated doses of antigen, to the responses of animals receiving only one injection of the full dose Ag, formulated in either a W/O emulsion or in saline. Apart from a regimen of fractionated, repeatedly administered, constant Ag doses, we also examined multiple injections of variant Ag dose-strength for their capacity to elicit Ag-specific antibodies.

### **Materials and methods**

**Preparation of vaccines.** All Ag formulations contained formalin-inactivated IBDV strain D78. Vaccine Ags used for repeated injection were diluted with 0.01 M Phosphate buffer, pH 7.2 without addition of any immunopotentiating agent. One emulsion was prepared using Mineral oil (Marcol 52, Exxon, USA) as the oil phase. The W/O emulsion was prepared by adding the aqueous phase into the oil phase, while mixing was performed with high shear forces at 20,000 r.p.m. using an Ultra Turrax Type T25 (IKA, Germany). The aqueous phase, containing the virus suspension was diluted with 0.01 M phosphate buffer, pH 7.2.

**Immunization of animals.** Groups of 8–10 three- to four-week-old specific pathogen free (SPF) female White Leghorn chickens, housed under clean conditions with access to water and standard commercial food *ad libitum*, were used for immunizations. In the case of repeated administration, a 0.1 ml/dose was administered

into the left and right breast muscle, respectively, every other day, for a period of 48 days. Two groups of chickens received a series of repeated vaccinations. One group (group 1), received a constant IBDV dose of 4 units for 25 repeated administrations and the second group (group 2) a decreasing dose during the first 4 administrations at a rate of 31, 15, 8 and 4 units/dose; the 5th - 25th administration contained 2 units/dose (Table 1). The total cumulative amount of Ag administered in both series of repeated injection was 100 units. Three reference groups were included in this study. One group received a single dose of 100 units an inactivated IBDV vaccine formulated in the W/O emulsion (group 4), while the second reference group received a single dose of 100 units IBDV diluted with 0.01 M phosphate buffer, pH 7.2 (saline; group 3). With the single dose injection, chickens received 0.5 ml in the breast muscle at time zero. In addition, one group received only 2 units of Ag in W/O emulsion (group 5).

To investigate the immunopotentiating effects of repeated injections, one special reference group was added to the experiment. This group received at time zero 100 units of IBDV diluted with 0.01 M phosphate buffer, pH 7.2 in a 0.1 ml dose, followed by 24 injections of placebo (0.1 ml of phosphate buffer), every other day, for a period of 48 days.

Blood samples for serological tests were taken at weeks 3, 6, 9, 12, 15 and 18 after vaccination. Blood samples were also taken at week 7, the time of the final administration of the repeated injection series.

Local reactions at the injection site were monitored at 18 weeks after vaccination. All experiments were approved by an independent Animal Welfare and Ethical Review Committee in accordance with the Dutch Law on Animal Experiments.

**Assays for serum antibodies.** Serum levels of IBDV-specific antibodies were determined by virus neutralization assay. Serial two-fold serum dilutions were prepared in microtitre plates and mixed with an equal volume containing 650TCID<sub>50</sub>/100 µl of IBDV. After pre-incubation, chicken embryo fibroblast (CEF) cells were added. For at least 5 days the cells were cultivated and then examined for the presence or absence of typical cytopathic effect (cpe). The titres of the test samples are expressed as means (log<sub>2</sub>) of the reciprocal of the highest dilution where all virus was neutralized.

**Statistical analysis.** Comparison of means of serum titres was performed with one-way analysis of variance (ANOVA) by comparing time point values between groups and by group analysis between time points (GLM) with least significant difference (LSD) as multiple comparisons test. All results were generated using SAS (version 8.2) software. Differences were considered significant if P was < 0.05.

## Results

**Safety of experimental procedures.** During the course of the study all immunized animals appeared healthy without notable clinical symptoms, although some immunization groups may have been “stressed”, to a limited extent, by injections every other day for a period of 48 days. Local reactions at the injection site were monitored at 12 weeks after vaccination. Only the vaccine based on W/O emulsion showed residues of the inoculum as expected (data not shown).

### **Effects of distinct antigen administration schemes on specific antibody**

**formation.** Results of the immunization efficacies measured as circulating virus-neutralizing antibody titres in the time after immunization are shown in Figure 1. Repeated injection of a constant, small dose of inactivated IBDV antigen (4 units), every second day, over a period of 48 days (group 1), evoked antibody responses that gradually increased over time. The rise in serum titres continued for two weeks after the end of antigen injections. Repeated injection of decreasing amounts of antigen (group 2), i.e. an initial high dose, followed by a gradual dose decline was more efficacious, when compared to repeated administration of identical Ag amounts of 4 units (group 1). After the end of the antigen injections the gradual decline in antibody titre was similar between groups. Administration of a single full antigen dose (100 units) in saline buffer at time zero evoked a more effective humoral immune response detectable at 3 weeks after immunization, with a slow decline in antibody titres during the 18 weeks of measurement (group 3).

To examine potential immunopotential as a result of local “danger” signals resulting from theoretical injection trauma at the site of inoculation, we tested the

effect of repeated saline (placebo) injections after a full dose (100 units) at day 0 and in another group after a minimal single dose of 2 units. This showed that additional administration of saline (placebo) injections every other day during a period of 48 days, stimulated no increase in antibody response, when compared to a single injection of 100 units of Ag (data not shown). A single injection of 2 units followed by repeated injections of saline on every other day gave no measurable titre (data not shown). Even when formulated in W/O emulsion, 2 units of Ag failed to induce a detectable antibody titre (group 5). However, the same W/O formulation improved antibody titres, approximately 4-fold ( $2 \log_2$ ) for 100 units of Ag (group 4), when given as a single dose at day 0. Interestingly, after about 7 weeks the kinetics of the serum antibody titre was comparable to that of the single dose administration without immunopotentiator and resembled the profile noted in an earlier study (Rijke *et al.*, 1995). Notably, statistical analysis revealed no significant differences in the kinetics of circulating antibody titres between groups immunized with Ag in saline only, including those receiving fractioned Ag dosing, at 6 weeks after primary immunization and later time points until the end of the experiment. However, data from Figure 1 suggest that Ag dose (high versus low) determine the acceleration / kinetics of the antibody response from 0 - 3 weeks, whereas Ag potentiated by the W/O emulsion affected the magnitude but not the kinetics. This suggests the importance of the adjuvant not in the form of a depot, but to function in an as yet undetermined facilitating fashion, for example increasing the number of activated B cells.

We conclude that repeated administration of small amounts of IBDV Ag results in a gradual increase of specific humoral immunity in White Leghorn chickens, although less effective when compared to a single dose vaccination of the

same amount of Ag formulated in a W/O emulsion. The in-vivo data of this experiment were in line with those of a similar experiment in SPF White Leghorn chickens performed earlier (data not shown).

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## Discussion

The requirements for vaccine Ag release from depot type delivery systems are poorly understood. The purpose of the present study was to obtain more information on the importance of a slow Ag release mechanism for induction of antibody responses in chickens. We therefore initiated investigations into the relationship between dose and time of in vivo Ag availability and specific antibody formation. Earlier studies of Herbert indicated that antibody responses obtained by injection of very small daily doses of Ag in mice could mimic the effectiveness of depot-forming adjuvants like W/O emulsions (Aucouturier *et al.*, 2001). Indeed, in our study we also found stimulation of the antibody-forming cells and a gradual rise in circulating antibody titres in White Leghorn chickens by repeated injection of tiny quantities of Ag. However, both the onset and the magnitude of the antiviral antibody titre was less pronounced when compared to that after injection of a full dose formulated in a W/O emulsion.

Our results are not completely in line with the findings of Herbert. The peak antibody response obtained by repeated injections was never as high as the antibody level obtained by a single administration of a full Ag dose in a W/O emulsion or in saline alone. We used a two-day time interval between the administration of Ag, which was given in the left and right breast muscle in an alternating injection schedule, instead of Herbert's daily dose regimen given subcutaneously. Also, we measured responses in poultry, while Herbert studied responses in mice. In addition, IBDV antigens in saline alone are capable of inducing high antibody responses, because virus particles with repetitive structures are more immunogenic when compared to ovalbumin (Bachmann *et al.*, 1993; Jegerlehner *et al.*, 2002; Wit de *et*



*al.*, 2004). Notably, the immunogenicity of the viral Ags proved to be dependent on the concentration of inactivated virus particles at the injection site following the first injection. The initial amount of IBDV in the repeated low dose (4 units) schedule was probably too low to induce prompt antibody formation. Repeated injections starting with a higher dose of 31 units were more efficacious, and just one dose of 100 units was most effective in antibody induction. A higher initial dose resulted in an earlier peak of antibody titres. However, at 6 weeks after primary immunization and at later time points no statistically significant differences between the levels of serum antibody titres were noted between these groups.

In general, Ag availability is considered necessary for the maintenance of Ag-specific antibodies in the circulation for a prolonged period of time. It is debatable whether Ag availability can only be achieved by prolonged or pulsed release of free Ag. Oil-based vaccines are very effective in maintaining Ags at the injection site. In vitro release studies of several W/O types of emulsions showed no detectable release of Ags during the first 3 weeks of measurement (Aucouturier *et al.*, 2001; Jansen *et al.*, 2005; Jansen *et al.*, 2006). Hence, the importance of a depot with a high concentration of stable Ags at the injection site should not be underestimated. As soon as Ag-specific antibodies have been formed, the sustained presence of Ag in draining lymphoid organ and at the injection site is likely to be an important factor for the duration of the response.

However, since initiation of adaptive immune responses takes place in lymphoid organs, Ags need to be translocated from the site of injection towards the local lymph node or the spleen, where interaction with adaptive T- and B cells allow the completion of T cell dependent B cell responses (Flarend *et al.*, 1997). Itano *et al.* (2003) describe this translocation of Ags in mice as two waves of antigen influx. The

first Ag influx is based on intact Ags that reach the T cell zone in the lymph node early after injection. The second wave of Ag influx occurs within 24h of injection and is based on Ag-presenting dendritic cells at the injection site that contain processed Ag and move towards the nearest draining lymph node via afferent lymphatics (Itano *et al.*, 2003). This suggests that a period of about one day of Ag persistence may be sufficient to evoke an augmented level of antibody production. This may explain the activity of depot-type delivery systems such as W/O-based emulsions and aluminium. Flarend *et al.* (1997) demonstrated that aluminium salts already start to dissolve within 1h of injection. Aluminium salts may maintain Ag at the site of injection for a relatively short, but sufficient, period of time.

Freund studied the role of the depot function in rabbits, by measuring antibody formation after surgical removal of the vaccine from the site of injection. Excisions performed between 30 min and 4h after injection resulted in a decrease of the immune response when compared to the response in animals in which the injected areas were not removed. However, excisions performed after one or more days did not seem to affect the antibody response (Freund, 1951).

W/O emulsion-based vaccines effectively maintain Ags at the injection site, enabling injection of less Ag when compared to non-adjuvanted vaccines, which makes them attractive vaccine vehicles from an economical point of view for the poultry industry (Aucouturier *et al.*, 2001). The observed immediate improvement of the overall magnitude of antibody titres by W/O emulsions early after immunization, when using a constant amount of Ag, indicates that W/O emulsions also act as direct immune system activating immunopotentiators, which may vary in efficacy depending on the type of oil used (Jansen *et al.*, 2005). The emulsion structure or carbohydrate molecules may facilitate, directly or through release of local mediators,

the recruitment of the desired type of antigen presenting cells (APC). Uptake of Ag by recruited APC may occur after endocytosis of Ag-containing W/O fragments, since very little antigen is released from the W/O depot in vitro and probably in vivo (Aucouturier *et al.*, 2001; Jansen *et al.*, 2005; Jansen *et al.*, 2006).

In conclusion, a combination of both prolongation of Ag residence together with direct immune stimulation makes W/O emulsions very effective vaccine vehicles to improve Ag-specific humoral responses. The critical spatio-temporal characteristics of the release of free Ag from the depot at the injection site remains to be defined (Schijns, 2000) and has to be studied in more detail.

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**Table 1:** Amounts of antigen (units) administered during the study

Group	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10 -48	Total amount of antigen	Vehicle
1	4 <sup>a</sup>	4	4	4	4	4 daily	100	saline
2	31	15	8	4	2	2 daily	100	saline
3	100	- <sup>b</sup>	-	-	-	-	100	saline
4	100	-	-	-	-	-	100	w/o emulsion
5	2	-	-	-	-	-	2	w/o emulsion

<sup>a</sup> units of antigen administered on each occasion

<sup>b</sup> no antigen administered

**Table 2:** *Statistically significant differences ( $p < 0.05$ ) between groups at different time points*

Statistical comparison between groups	1-2	1-3	1-4	1-5	2-3	2-4	2-5	3-4	3-5	4-5
3 weeks after vaccination	* <sup>a</sup>	*	*		*	*	*		*	*
6 weeks after vaccination	<sup>b</sup>	*	*	*	*	*	*		*	*
7 weeks after vaccination		*	*	*		*	*		*	*
9 weeks after vaccination		*	*	*		*	*	*	*	*
12 weeks after vaccination		*	*	*		*	*	*	*	*
15 weeks after vaccination		*	*	*		*	*	*	*	*
18 weeks after vaccination			*	*	*	*	*	*	*	*

<sup>a</sup> difference significant ( $p < 0.05$ )

<sup>b</sup> no significant difference between groups



**Figure Legends**

**Figure 1.** *Serological responses expressed as group means of IBDV specific neutralizing antibody titres (log<sub>2</sub>).*

The arrow indicates the termination of repeated antigen administration. Statistical analysis revealed significant differences ( $p < 0.05$ ) at time points indicated in Table 2. Statistical analysis between time points within groups revealed no significant differences for any group, except for group 1 between weeks 3 and 9 and between weeks 3 and 18, as well as within group 5 between weeks 7 and 15 and between weeks 15 and 18.