Evaluation of safety and immune response induced by several adjuvants included in *Pasteurella multocida* vaccines in chickens

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SUMMARY

The objective of the present study was to investigate the safety of several vaccine preparations associating killed *Pasteurella multocida* antigens and different adjuvants and their immune efficacy in chickens in comparison with a commercial vaccine. Vaccine efficiency was assessed by measuring serum antibody titres 4 and 8 weeks after a single vaccination and adverse reactions at the injection site were monitored at necropsy by macroscopic examination and eventually by histological examinations. The vaccines formulated with oil adjuvant Montanide ISA 70, ISA 774 and W/O emulsion based on Tween / span induced a strong immune response against *Pasteurella multocida*. No antibody response was obtained using aqueous nano-particle compound (Montanide IMS 1112). The intensity of local side effects according to the vaccine formulations was dependent on oil content, HLB (Hydrophilic-Lipophilic Balance) and quality of surfactants: particularly, montanide ISA 774, an adjuvant with mixed mineral and non-mineral oils, appeared to be weakly reactogenic.

Keywords: Vaccination, chicken, adjuvants, *Pasteurella multocida.*

RÉSUMÉ

Evaluation de l'innocuité et de la réponse immunitaire induite par des vaccins contre *Pasteurella multocida* contenant différents adjuvants chez le poulet

L'objectif de cette étude était de tester l'innocuité et la réponse immunitaire induite par différents adjuvants entrant dans la composition d'un vaccin inactivé contre *Pasteurella multocida* chez le poulet, en comparaison avec un vaccin commercial. L'efficacité a été évaluée en quantifiant les titres en anticorps sériques obtenus 4 et 8 semaines après une seule administration de vaccin. Les réactions à la vaccination au site d'injection ont été appréciées macroscopiquement et éventuellement par un examen histopathologique. Les vaccins contenant les adjuvants Montanide ISA 70 et ISA 774 ainsi qu'une émulsion tween / span ont induit une forte réponse en anticorps vis-à-vis de *Pasteurella multocida* alors qu'avec le vaccin contenant des nano-particules dispersées en milieu aqueux (Montanide IMS 1112) aucune réponse en anticorps n'a été observée. L'intensité des réactions locales aux vaccins était liée à différentes caractéristiques des adjuvants utilisés : la nature de l'huile et du surfactant ainsi que l'équilibre hydrophilie-lipophilie : l'adjuvant Montanide ISA 774 contenant un mélange d'huiles minérale et non minérale a, en particulier, provoqué peu de réaction locale.

Mots-clés : Vaccination, poulet, adjuvant, *Pasteurella multocida.*

Introduction

Induction of protective immunity using inactivated antigens requires their association with effective adjuvants which are selected according to various criteria in order to get the best balance between efficacy and safety.

When killed vaccines are used in birds, animals are injected with antigens incorporated in a classical water-in-mineral oil emulsion which is often accompanied by local side-effects such as granuloma and sterile abscess formation. The quality of antigens and surfactants as well as the nature of the oil are responsible for these side-effects. Several alternatives exist to avoid such local reactions like reducing the viscosity of the emulsion with specific surfactant systems or modifying the ratio between oil and water [10]. Non mineral oils which are better tolerated can also be used but to the detriment of efficacy. A mix of mineral and non mineral oils can then be a good compromise. Other types of adjuvants like multiphase emulsions or nano-particles are usually well tolerated and used in vaccines dedicated to sensitive animals [1].

We were interested in testing such products in one avian species in order to identify alternatives to the water-in-mineral oil adjuvants. In this study, efficacy and safety of several preparations formulated with different adjuvants were assessed in comparison with an inactivated commercial vaccine. For that, we have chosen fowl cholera as a study model since (i) this disease is of economic importance worldwide (ii) several inactivated vaccines are frequently used under field conditions and (iii) there is a strong correlation between antibody response and level of protective immunity (reviewed in [8])

Material and Methods

ANIMALS

A total of 120 ISA Brown layers, 16 week old at the beginning of the experiment, were bought from a commercial laying farm. They were maintained in an open space with food and water *ad libitum* at the Ecole Nationale Vétérinaire de Nantes (France).

ANTIGENS, ADJUVANTS AND VACCINES

The antigens included in the vaccine preparations resulted from inactivation of a *Pasteurella multocida* strain (serotype 3) isolated from a commercial duck with clinical signs of cholera. The antigens were produced in brain-heart infusion (BHI) broth by incubating the bacterial strain for 18 hours at 37°C. Following incubation, the culture was inactivated by 0.2% formalin and heated at 60°C for one hour. Vaccines were prepared by manually emulsifying the antigen solution in the different adjuvant formulations tested (Table 1) at ratios of 7:3 (standard and experimental Montanide ISA and water in oil (W/O) emulsion based on Tween / span) or 1:1 (Montanide IMS 1112). The final concentration of *Pasteurella multocida* antigen was 5.5 10^7 CFU per vaccine before inactivation. The adjuvants used have been manufactured by SEPPIC, Vaccine & Injectable Business Unit, Paris, France.

IMMUNISATION OF CHICKENS

Chickens were randomly divided into 6 groups of 15 animals and were immunised via the subcutaneous route in the neck region with 0.5 ml of each vaccine preparation. One group was vaccinated with a commercially available *Pasteurella multocida* killed vaccine (AVIPASTOVAX®, Mérial, Lyon, France) whereas the other groups were vaccinated with the different experimental preparations based on the five adjuvants tested. A control group of 30 animals injected with antigens alone (no adjuvant) was included in the study.

DETERMINATION OF ANTI- *PASTEURELLA MULTOCIDA* ANTIBODY SERUM TITRES

Blood samples from all birds were collected before vaccination (t = 0) then 4 and 8 weeks after vaccination. Blood was allowed to clot at room temperature, samples were centrifuged (2 400 g, 5 minutes at room temperature) and sera were carefully harvested and stored at –20°C until assayed. Sera were tested for antibodies to *Pasteurella multocida* by an enzyme-linked immunosorbent assay (ELISA Kirkegaard and Perry Laboratories (KPL) Gaithersburg, MD) according to the manufacturer's recommendations. Antibody titres were expressed as the 2-log of the regression coefficient of the plot of optical density versus concentration. Sera were considered positive when antibody titre was higher than 149.

EVALUATION OF SAFETY OF VACCINE PREPARATIONS

After vaccination, birds were monitored daily and clinical signs were recorded. Eight weeks after vaccination, birds were euthanatized, necropsied and evaluated for a local lesion at the injection site. When gross changes were present, histological findings were obtained from subcutaneous tissue fixed in 10% buffered neutral formalin and stained with haematoxylin and eosin.

STATISTICAL ANALYSIS

Comparison between groups was based on two-way ANOVA on the ranks of crude titre values (PROC GLM, SAS Institute Inc., 1989) followed by a Tukey's test. Differences were considered as significant when p values were less than 0.05.

Results

ANTIBODY RESPONSES TO VACCINE PREPARATIONS

Descriptive statistics were provided before and 4 and 8 weeks after vaccination in groups 1 to 7 (Table 2). As serum antibody titres were not normally distributed, minimum, quartile 1, median, quartile 3 and maximum values were given and the statistic analysis was based on the ranks of titre values. Before vaccination, some animals (21/120 i.e. 17.5%) exhibited serum titres of anti-*Pasteurella multocida* antibodies higher than 150 and antibody titres varied from 3 to 1067 in the overall bird population.

At 4 and 8 weeks after vaccination, the antibody response induced by all adjuvants was significantly higher than that of control group (no adjuvant) except for IMS 1112 (Table 2). Although the protection levels were not statistically significant between the different vaccine preparations (excepted the vaccine with IMS 1112), the classification of protective vaccines based on ranks of antibody titres in terms of decreasing efficiency was at 4 weeks after vaccination the following: ISA 70 HLB 7.0; W/O emulsion based on Tween / span; ISA 70 HLB 7.5; ISA 774 and finally the commercial vaccine. At 8 weeks after injection the ranking was the following: ISA 70 HLB 7.0; W/O emulsion based on Tween / span; ISA 774; Commercial vaccine and ISA 70 HLB 7.5. No significant decrease of antibody titres was observed between 4 and 8 weeks after vaccination.

ADVERSE REACTIONS TO VACCINATION

No chicken included in this study died or developed any clinical signs after vaccination. All chickens were checked for the presence of gross lesions at time of necropsy. Pathological changes observed at the injection site 8 weeks after vaccination are shown in Table 2 (III). When present, macroscopic abnormalities consisted in thickening of the subcutaneous tissue and no abscess could be observed. The number of birds showing lesions varied between 0 and almost all animals of the group depending on vaccine prepa-

rations. No lesion was observed on animals vaccinated with Montanide IMS 1112, experimental Montanide ISA 70 (HLB value (Hydrophilic Lipophilic Balance): 7.5) as well as in the control group (inoculated with antigen without adjuvant). Among birds inoculated with Montanide ISA 774, few of them exhibited local adverse reactions. Lesions were noted in half of the animals vaccinated with commercial vaccine and standard Montanide ISA 70 (HLB value: 7.0). The majority of birds inoculated with a W/O emulsion based on Tween / span exhibited local adverse reactions.

At histological examination vaccine preparations were shown to induce inflammatory lesions consisting of cysts and granulomas with infiltration of macrophages and plasma cells around them. Some lesions were surrounded by fibrosis.

Adjuvant	Oil	Surfactant	HLB* (Polarity)
Standard Montanide ISA 70	Mineral	Mannide oleate	7.0
Experimental Montanide ISA 70	Mineral	Mannide oleate	7.5
Montanide ISA 774	Mineral + metabolisable	Mannide oleate	5.0
Tween / span formulation	Mineral	Sorbate / polysorbate	7.0
Montanide IMS 1112	Liquid nano-particles	Aqueous dispersion	> 10

* HLB: Hydrophilic Lipophilic Balance calculated according to the GRIFFIN formula [3].

TABLE 1: Description of the different adjuvant formulations tested.

Group and vaccine formulation	n*	Minimum	Quartile 1	Median	Quartile 3	Maximum	Mean	Local reactions
Montanide ISA 70 HLB 7.0								
t = 0	15	14	23.5	33	52	237	86	
t = 4 weeks	14	80	1 024	1 279.5	4 364	31 611	8 888[a]	
t = 8 weeks	13	14	244	1 629	5 267	14 423	9 125[a]	6 / 13
Montanide ISA 70 HLB 7.5								
t = 0	15	34	42	63	111	206	54	
t = 4 weeks	14	43	1 298	4 907	18 890	27 685	5 346[a]	
t = 8 weeks	14	84	2 339	6 829	6 829	13 303	3 706[a]	0 / 14
Montanide ISA 774								
t = 0	15	3	20	87	93	156	68	
t = 4 weeks	15	134	423	1 975	4 396	22 027	5 219[a]	
t = 8 weeks	13	50	532	2 500	5 505	32 732	5 763[a]	3 / 13
W/O emulsion (Tween / span)								
t = 0	15	15	30	46	188	426	108	
t = 4 weeks	15	272	732	2 285	8 826	19 424	5 562[a]	
t = 8 weeks	14	205	1 079	8 909.5	11 690	13 960	7 252[a]	12 / 14
Montanide IMS 1112								
t = 0	15	9	30	45.5	87	203	67	
t = 4 weeks	13	16	30	65	160	395	128[a]	
t = 8 weeks	12	11	26	55	109	156	69[a]	0 / 12
Commercial vaccine								
t = 0	15	11	46.5	102.5	127	405	127	
t = 4 weeks	11	51	171	1 921	4 342	5 642	2 424[a]	
t = 8 weeks	10	41	64	807	3 972	16 684	3 466[a]	5 / 10
Antigens (no adjuvant)								
t = 0	30	5	41.5	66.5	123.5	1 067	131	
t = 4 weeks	30	4	57	84.5	136	1 573	156[b]	
t = 8 weeks	30	0	17	29.5	73	1 338	103[b]	0 / 30

N* Number of chickens tested. In some groups, it decreased with time because of loss of identification system. Different superscripts [ab] in the same column indicate significant differences between groups ($p < 0.05$) for a given sampling time (4 or 8 weeks post vaccination) using the test based on ranks.

TABLE 2: Serum antibody titres against *Pasteurella multocida* obtained in ISA Brown layers before (t = 0) and 4 and 8 weeks after vaccination with different vaccine formulations and local adverse reactions at the injection site observed 8 weeks after vaccination against *Pasteurella multocida* with different vaccine formulations.

Discussion

Several adjuvants were investigated in chickens for their ability to induce both protective immune response and weak / mild adverse reactions. The field of vaccine strategies applied to poultry depend on both animal species and production type as well as on the disease to prevent. Concerning prevention of cholera, common vaccination schedules consist of administering a killed vaccine twice or three times. In this study, birds received a single injection in order to allow discrimination between vaccines. Indeed, it has been previously established that the adjuvant influence on immunity is essential during the first administration of vaccine [4].

All the adjuvants tested here allowed preparation of stable emulsions with a good injectability. Efficacy of the vaccine preparations was assessed by measuring serum anti-*Pasteurella multocida* antibody titres. Previous studies have shown that although cell-mediated immunity is of importance in poultry immunity against these bacteria, ELISA antibody titres highly correlated with survivability following virulent challenge [6]. In this study, antibody titres obtained after vaccination with bacterial antigens associated with all the adjuvants tested except IMS1112 were within the range of response induced by commercial vaccine whose efficacy has been already established.

Before vaccination almost all chickens exhibited titres of serum antibodies against *Pasteurella multocida*. Nevertheless, antibody titres markedly increased 4 and 8 weeks after vaccination but greatly varied within a same group, the high inter-individual variability of immune response being related to the presence of some non-responders in each group. Contrary to field conditions where large numbers of animals are simultaneously vaccinated and thus missing of some individuals is likely, all animals are supposed to have been injected in this study. No increase of antibody titre was observed in the control group over time meaning that animals have not been unintentionally exposed to the bacteria during the experiment. Indeed, vaccinated and control chickens have been housed together for all the study period.

Chickens were killed 8 weeks post injection in order to evaluate local adverse reactions since a previous study has shown that at that time inflammatory reactions at injection site started to regress [10]. Consequently, this protocol did not allow assessment of the duration of immune protection induced by the vaccines tested. When present, pathological changes at injection site were almost similar for all vaccine preparations and consisted of proliferation of epithelioid cells and macrophages around cysts, evidencing inflammatory reaction. However, there were quantitative differences between vaccinated groups since the number of animals per group exhibiting lesions varied from 0% to 85.7%. As antigen amounts in commercial vaccine and in experimental formulations were comparable (10^8 to 10^9 CFU / ml) differences in the induction of a local reaction are probably due to the adjuvant effect. The formula based on Tween / span associated with mineral oil with HLB 7.0 (traditionally used in poultry vaccines) was found the most reactogenic in the present experiment. Using the same mineral oil best results were obtained (i) with mannide oleate HLB 7.0 instead of Tween / span as surfactant and (ii) by increasing HLB value of mannide oleate to 7.5. Mannide oleate surfactants dedicated to injectable applications are well defined and controlled products in terms of chemical values and purity compared to multi purpose classical polysorbates as Tween / span (internal SEPPIC data / not published). A key issue of this study was that even for controlled mannide oleate surfactants a low increase in polarity resulted in unexpected improvement of vaccine safety. It was previously established that a shift in HLB value had an influence on emulsion stability that can be different depending on storage temperature [9]. However, little is known about influence of stability of emulsions at physiological temperature on both efficacy and safety since it was established in poultry that a slow release rate of antigens induces a stronger immune response [2]. Another way for improving local tolerance was to lower the content of mineral oil as it was done in Montanide ISA 774 where a part of mineral oil was replaced by metabolisable oil from vegetable origin.

A classification of vaccine formulas according to their polarity indicated that the highest HLB values induced a best local tolerance except the Tween / span formulation. However, in case of Montanide 774 (HLB value: 5), this parameter is partly balanced by replacement of the mineral oil by a non-mineral one. Further studies are needed to confirm the HLB influence on safety of vaccine formulas. Nevertheless, a significant increase of HLB value is not possible without impairing vaccine stability. Moreover, the IMS 1112 adjuvant (HLB value > 10) associated with *Pasteurella multocida* antigens failed to induce any protective immune response against fowl cholera in this study. It belongs to another category of adjuvants consisting of nano-particles combined with an immuno-stimulating compound. Such a product has proved safe and effective in vaccination trials against swine atrophic and bovine anaplasmosis rhinitis [5, 7]. The results obtained here emphasize the fact that each adjuvant has to be tested in the target species in association with specific antigens since adjuvanticity appears to be not universal.

More studies are required in order to understand the mechanisms of action of adjuvants and to predict both immune response induced and potential local and / or systemic reactogenicity. These predictions can help manufacturers to improve their products having in mind the possible use of them and the obligations linked with this use. This study proved that nano-particles adjuvants are ineffective in poultry whereas Montanide ISA 70 appeared to exhibit both efficiency and safety. Moreover, Montanide ISA 774 where the mineral oil is partly replaced by metabolisable oil appears to be relevant to adapt adjuvant effect to antigen reactogenicity and provide both immune efficiency and safety. This type of formulation where the ratio mineral oil / non mineral oil is an alternative to water-in-mineral oil emulsions would be particularly indicated for vaccination with crude reactogenic antigens.

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